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Type 1 diabetes, glucocorticoids and the brain: a sweet connection

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Type 1 Diabetes, Glucocorticoids and the Brain: a sweet connection

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The studies presented in this thesis were performed at the Division of Medical Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR) and Leiden University Medical Center (LUMC), The Netherlands. The studies described in the chapter 4 were carried out at the Institute of Biology and Experimental Medicine (IBYME) in Buenos Aires, Argentina. The support by NWO-WOTRO and the Royal Netherlands Academy of Arts and Sciences is greatly acknowledged.

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"El trabajo tenaz y concentrado es una fuerza poderosa, como la llama de un soplete que aplicado en un punto fijo de la plancha más dura de acero acaba por ablandarla y taladrarla, mientras que si se pasea de un punto a otro no alcanza ni a entibiarla".

Bernardo Alberto Houssay, Buenos Aires (1884-1971)

A mi familia

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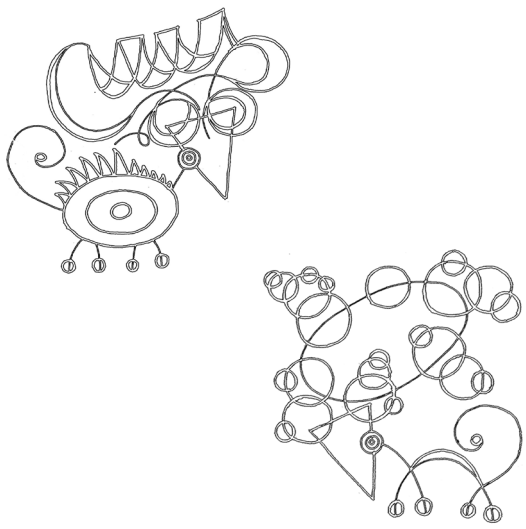
CHAPTER 1

GENERAL INTRODUCTION



Outline

1. Type 1 diabetes

The earliest known record of diabetes dates from 1552 B.C., when the 3rd Dynasty Egyptian papyrus by physician Hesy-Ra mentioned polyuria (frequent urination) as a symptom of the disease. Over the years, diabetes was described and studied by Egyptians, Greeks, Chinese, Indians, English, Frenchs, Germans, Czechs, Italians, Canadians, Americans, and others. However, was not until the last century, in 1959, that the two major types of diabetes were recognized: type 1 (insulin-dependent) diabetes and type 2 (non-insulin-dependent) diabetes.

Nowadays diabetes affects over 150 million people worldwide with this number expected to double by 2025; about 90% cases of diabetes are type 2 (Zimmet *et al*, 2001). However, the fraction of type 2 diabetics in different parts of the world varies substantially, almost certainly for environmental and lifestyle reasons, though these are not known in detail. Since type 2 diabetes is not the topic of this thesis, it will not be described at length.

Diabetes mellitus type 2, also called type 2 diabetes, Non Insulin Dependent Diabetes Mellitus (NIDDM) or Adult diabetes, is a metabolic disorder that is primarily characterized by insulin resistance, relative insulin deficiency, and hyperglycemia, and is presently of unknown etiology although there is a strong inheritable genetic connection. About 55% of type 2 are obese (Eberhart *et al*, 2004) -chronic obesity leads to increased insulin resistance that can develop into diabetes, most likely because adipose tissue is a source of chemical signals (hormones and cytokines). Conversely, type 2 diabetes causes obesity (Camastra *et al*, 1999). Additional factors found to increase risk of type 2 diabetes include aging (Jack *et al*, 2004), high-fat diets (Lovejoy, 2002) and a less active lifestyle (Hu, 2003).

Diabetes mellitus type 1, also known as type 1 diabetes (T1D), Insulin Dependent Diabetes Mellitus (IDDM) or Juvenile Diabetes, is an autoimmune disease that results in the permanent destruction or damage of insulin producing beta-cells in the islets of Langerhans of the pancreas. Destruction of these cells leads to insulin deficiency. Therefore, T1D is lethal unless treatment with exogenous insulin via injections replaces the missing hormone. Although, the clinical consequences of the disease have been extensively investigated, the exact cause(s) of T1D are not yet fully understood. Genetic and environmental factors have been suggested to contribute to the etiology of T1D along with other factors (see Box 1 on page 14). So far, the research core on diabetes has focused on the peripheral endocrinology and nervous system. Nowadays, the impact of diabetes on the central nervous system (CNS) is highly recognized but it was not always the case until few decades ago. To study disease initiation, progression, and treatments without exposing humans to unnecessary and potentially unethical risks animal models have been developed. Animal models have contributed important knowledge regarding the study of diabetes. The physiology of mice, rats, and other animals is remarkably conserved in comparison to the human condition. Broad spectrum of animal models of T1D have become available over the last 40 years. They comprise spontaneous models, in which disease develops unprovoked, and experimental models induced by various types of intervention (Table 1, page 15).

1.1. Animal models of diabetes

1.1.1. Spontaneous Models

The two major models used are the so-called Bio Breeding (BB) rats and the Non Obese Diabetic (NOD) mice, which develop the disease with similarities to human T1D. These animals derived from inbreeding over many generations by selecting for hyperglycaemia. As a result, many genes and phenotypes have been enriched, but not all will be relevant to the pathophysiology of diabetes, either in rodents or in humans. It is noteworthy that a main advantage of these models is the possibility to study the pre-diabetic state, which is impossible in humans. Other models comprise the Long Evans Tokushima Lean (LETL) rat and the LEW.1AR1/Ztm- iddm rat (Table 1). For sake of clarity, only the model used in the current thesis will be described below.

1.1.1.a. The NOD mouse

The NOD mice were first used in the study of cataract development (i.e. JcI-ICR mouse) (Makino *et al*, 1980). Insulitis, which is the lymphocytic infiltration of the islets of Langerhans, is present by the time mice reach 4-5 weeks of age. This state is followed by beta-cells destruction and ultimately leads to a drastic decrease in circulating insulin. In the pre-diabetic state (4-5 weeks of age), NOD mice show lower glycemia and higher insulinemia in response to a glucose tolerance test compared with C57Bl/6 control strain (Amrani *et al*, 1998). Frank diabetes typically begins between 12 and 30 weeks of age. Unlike human T1D, ketoacidosis (metabolic acidosis is caused by high concentrations of ketone bodies and breackdown of fatty acids) is relatively mild and affected animals can survive for weeks without the administration of insulin. In addition and in contrast to the findings of most studies in humans, there is a larger gender difference with 80% of females, but only 50% of males developing diabetes in some colonies (Atkinson and Leiter, 1999). This variation is not surprising knowing that sex steroids are part of the mechanisms underlying the well-recognized immune sexual dimorphism, which is particularly evident in autoimmune diseases (Ansar Ahmed *et al*, 1985). Moreover, NOD diabetes can be modulated not only by multiple immunotherapeutic agents (Bach 2002), but also by various other factors, including melatonin, insulin growth factor-1 (IGF-1), leptin, insulin and drugs modulating its secretion or sensitivity, and environmental factors such as temperature fluctuations, variations of protein and carbohydrate intake, and stress.

Stressful life events and diabetes onset linkage have been reported in clinical and experimental studies (Homo-Delarche *et al*, 1991; Djarova and; Dube, 1998.). As part of the endocrine response to stress, glucocorticoids exert well-known anti-inflammatory and immunosuppressive actions but also act as counterregulatory hormones inducing hyperglycemia. Therefore, in T1D, glucocorticoids might have both potentially beneficial and deleterious effects.

1.1.2. Experimental models

Most of the experimentally induced models correspond to highly artificial situations far from the conditions in which spontaneous disease develops. However, they have made possible remarkable progresses in understanding the pathogenesis of T1D. Chemically-induce, transgenic, and immunomanipulated mice are among these models. In the following section, the experimental model used in the present thesis is described.

1.1.2.b. Chemically induced T1D: Streptozotocin-induced diabetes mice

Pharmacological methods of inducing T1D by damaging the pancreas also exist. These include the administration of toxins such as streptozotocin (STZ) (Junod *et al*, 1969) and alloxan. Streptozotocin is a glucosamine–nitrosourea compound isolated from *Streptomyces achromogenes* with broad-spectrum antibiotic and anti-neoplastic activity (Bono *et al*, 1976). It is a powerful alkylating agent that has been shown to interfere with glucose transport (Wang and Gleichmann, 1998), glucokinase function (Zahner and Malaisse, 1990) and induce multiple DNA strand breaks (Bolzan and Bianchi, 2002). It is taken up into the insulin-producing beta-cells of the islets of Langerhans via the GLUT-2 glucose transporter (Schnedl *et al*, 1994). The GLUT-2 glucose transporter is absent at the blood–brain barrier (Kumagai, 1999), thus excluding direct effects of STZ on the brain following systemic administration. A single large dose of STZ can produce diabetes in rodents, probably as a result of direct toxic effects. Alternatively, multiple small doses of STZ are used (e.g. 40 mg/kg on five consecutive days) to study the immunological pathways that lead to insulitis and cell death (Mensah-Brown *et al*, 2002; Holstad and Sandler, 2001). STZ-diabetic rodents are hypoinsulinaemic, but do not require insulin treatment to survive. Blood glucose levels typically are 20-25 mmol/l, which is 5 fold over normal concentration. In rodents, hyperglycemia induces an insulinopenic (lack of insulin) diabetes in which immune destruction plays a role, as in human T1D.

Box 1: Etiology of type 1 diabetes

The origin of the autoimmune process that leads to type 1 diabetes (T1D) involve genetic predisposition (as T1D is known as a hereditary disease on basis of the relatively high rate of familial transmission (1)) and environmental factors, and their interactions, which creates the conditions required for disease onset. The patterns of familial transmission, combined with data from animal models, indicate that the determinism of T1D is polygenic and multifactorial. The search predisposition genes is complex, especially as most if not all predisposition genes appear to be basically "normal" i.e. without mutations or deletions. A fortuitous combination of these genes, together with permissive or triggering environmental factors, provokes the disease. Each of these genes may be present in a large proportion of healthy subjects (notably the patient's nondiabetic relatives).

Evidence for the role of environmental factors:

Several lines of evidence point to a major role of environmental factors in the pathogenesis of T1D. First, more than 60% of identical twins are discordant for the disease, and it is quite unlikely that this is due to differential somatic rearrangement of T cell receptors. Second, disease frequency varies enormously from country to country (2), and these differences cannot simply be explained by ethnic genetic differences since migrants from countries with a low T1D frequency to countries with a high frequency are more susceptible than their compatriots (3). Intriguingly, northern countries are more exposed to the disease than southern countries (2); it will be critical to discover the factor(s) responsible for this North/South gradient. Third, a number of apparently nonimmunological interventions can increase or decrease the disease rate in animal models: specific diets (low essential fatty acid (4) or protein intake (5, 6)) and several viral infections (7-11) can reduce disease susceptibility in spontaneous models of T1D, the NOD mice and the BB rats, while Kilham's virus (12) and cow's milk (13,14) can increase it in BB rats. These factors, particularly viral infections, probably explain the variations in disease frequency found between NOD colonies (15). Not only do environmental factors seem to influence T1D onset, they can also apparently alter the course of the disease. These factors can be shared by the whole population (climatic factors, hygiene, etc.), or by a given family (e.g. eating habits), or be specific to the individual (e.g. travels and sexual partners). Several studies have focused on many potential environmental factors involved in the etiology of T1D, such as viruses (16-18), bacteria's (19-22), toxic agents (23, 24), food constituents (5, 6, 13, 14), stress (25-28). These factors essentially modulate the expression of predisposing genes, either positively (predisposing factors) or negatively (protective factors). In the case of triggering factors, disease onset is directly related to the encounter with the environmental factor (usually single and limited in time), which can then be considered as the cause of the disease. In the "modulation" hypothesis, the disease can only appear in the fraction of the population at genetic risk and it is on this population that environmental factors (usually multiple and chronic) exert their positive or negative effect. The available data suggests that T1D is of the second type.

Table 1. Animal models of type 1 diabetes

Spontaneous models

1. NOD mouse: inbreed strain. Develop Type 1A-Immune Mediated Diabetes. Autoimmune etiology that is heavily influenced by both genetics and environment (1).
2. BB rat: inbreed strain. Diabetes in BB rats is also an autoimmune disorder. Substrain BB/Wor has profound T-cell lymphopenia (condition in which there exists an abnormally low number of lymphocytes in the blood) (2).
3. Long Evans Tokushima Lean (LETL) Rat: autoimmune T1D (3)
4. LEW.1AR1/Ztm- iddm rat: autoimmune T1D, spontaneous mutation within a Mhc-congenic LEW.1AR1 colony (4).

Experimental models:

Transgenic

1. T Cell Receptor (TCR) Tg (transgenic) Mouse: many cell clones isolated from the spleens of diabetic NOD mice, pancreas of pre-diabetic NOD, islet-transplanted diabetic NOD mice, and from islets of NOD mice. Many of these clones have been utilized to produce TCR transgenic (Tg) mice on various backgrounds (5)
2. "Humanized" Mice: transgenic expression in mice of human genes (6).

Chemically-induced

1. alloxan (7)
2. streptozotocin (8)

Immunomanipulation

1. thymectomy performed within 2 days after birth can induce a flourishing state of autoimmunity in mice (9).
2. adult thymectomy and sublethal irradiation (10, 11).
3. athymic rats with transfer of normal spleen cells (12).

2. Hypothalamic-pituitary-adrenal axis and type 1 diabetes

2.1. HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis refers to a complex set of homeostatic interactions between the hypothalamus (brain area); the pituitary gland (structure located below the hypothalamus), and the adrenal glands (small pair of pyramidal organs located on top of the kidneys). The HPA axis regulates responses to stress and modulates various body processes including growth, metabolism, immune response, mood, reproduction, sexuality, and energy balance. The core of the HPA axis is the paraventricular nucleus of the hypothalamus (PVN). The PVN contains neuroendocrine neurons, the so-called parvocellular neurons, which synthesize and secrete vasopressin (AVP) and corticotropin-releasing hormone (CRH). These two peptides can act in synergy on the anterior lobe of the pituitary gland to stimulate the secretion of the adrenocorticotropic hormone (ACTH) from corticotrope cells. In turn, ACTH enters peripheral circulation where it reaches the adrenal cortex to induce glucocorticoid hormones production (cortisol in humans, corticosterone in rats and mice). Glucocorticoids exert a negative feedback on the PVN and pituitary to suppress CRH and ACTH production, respectively.

Corticosterone is a major stress hormone and has effects on wide arrays of tissues in the body, including the brain. In the brain, corticosterone acts via two types of receptors - mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). These receptors are widely expressed throughout the brain by many different types of cells including neuron and glia. MR and GR have different affinities to glucocorticoids (GCs) with MR showing a greater affinity (10 fold higher) than GR. As a consequence, MR is fully occupied under basal circulating levels whereas GR becomes occupied only when glucocorticoids levels rise above normal. One important target of glucocorticoids is the hippocampus, an area of the limbic system that plays a critical role in memory, learning and spatial navigation. This structure is a major modulator of the HPA axis; hippocampal MR controls the inhibitory tone of this limbic structure on the HPA axis in terms of basal reactivity (Reul *et al*, 2000). This effect of GCs via MRs is modulated by GRs that become progressively occupied after stress and during the circadian rise of GCs. Therefore, predominant MR activation maintains hippocampal excitability and, through inhibitory projections to the PVN, basal HPA activity. Conversely, with rising GCs concentrations, GR activation suppresses the hippocampal output, resulting in a disinhibition of PVN neurons (de Kloet *et al*, 1998). In summary, a deficiency in MR is predicted to allow more GC release, thus leading to more pronounced GR-mediated effects. Therefore, the functions mediated by both receptor types are linked, and the balance in MR- and GR-mediated effects is important in the HPA regulation.

2.1.1. Stress concept

Stress is the disruption of homeostasis through physical or psychological stimuli. Internal

or external potential disturbances (stressors) activate two systems that serve to normalize the disturbed functions: the rapid sympatho-adrenomedullar system and the slow-acting HPA axis. The activation of the sympathetic branch result in the release of stress hormones including adrenaline from the adrenal medulla. Therefore, activation of noradrenergic neurons leads to temporarily elevated noradrenaline (NA) levels in specific areas of the brain resulting in functional changes of neurons carrying NA receptors. Activation of the HPA system, leads to increase GCs release from the adrenal cortex, which in turn will act in the brain at those sites where its receptors are enriched (Figure 1) (Joëls *et al*, 2007).

Sympathetic nervous output produces the fight-or-flight response, causing the body to divert blood flow to large muscles as the body prepares to run away from or fight something. Lower blood flow is then directed to the digestive system and other organs that do not assist in flying or fighting. Some stressors can cause continual sympathetic nervous system activation with very little opportunity for the parasympathetic nervous system to be activated. The activation of the parasympathetic system stops the fight-flight responses.

Experimental studies have investigated many different types of stressors, and their effects on the HPA axis in many different contexts. Analysis of the literature suggests that different classes of stressor employ different stress circuits. Severe physiologic ("systemic") stress appears to trigger brainstem/circumventricular organ systems that project directly to the PVN. In contrast, stressors requiring interpretation with respect to previous experience ("processive" stressors) reach the PVN by way of multisynaptic limbic pathways. Stressors of the latter category may thus require interaction with homeostatic information prior to promoting an HPA axis response. The HPA stress response thus appears to be a product of both the physiologic importance of the stimulus and the specific pathways a given stimulus excites (Herman *et al*, 1996). The activation of the HPA axis will ultimately trigger GCs secretion. In healthy condition this highly reactive system will turns on and off its responses to stressors. However, if adaptation to stress fails, the stress system responds slowly, or the stress reactions persist, circulating GC levels remain elevated for a prolonged period of time and an enhance vulnerability to disease for which the individual is predisposed may occur (de Kloet and de Rijk, 2004).

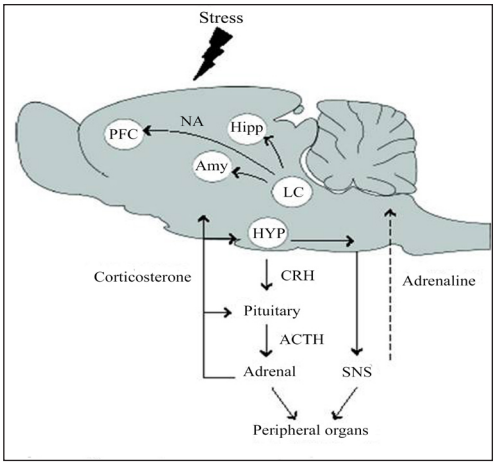


Figure 1: *Brain regions activated after stress exposure (Amy: amygdala, Hipp: hippocampus, PFC: prefrontal cortex) and output of these areas through the hypothalamus (HYP). A resulting activation of the fast acting sympatho-adrenomedullar system (right) and the slower acting HPA axis system (left) will affect the function of peripheral organs and feed back to the brain via adrenaline and corticosterone, respectively. Adrenaline will finally rise central release of noradrenaline leves from the locus coeruleous (LC), reaching again the amygdala, prefrontal cortex and hippocampus among other areas. Corticosterone will act on brain areas where its receptors are enriched.*
SNS = sympathetic nervous system; ACTH = adreno-corticotropin hormone; CRH = corticotropin releasing hormone.
Reprinted with permission from Joëls and Krugers, Neural Plasticity 2007.

2.2. Hypothalamic-pituitary-adrenal axis alterations in type 1 Diabetes

When T1D develops, the insulin producing beta-cells in the islets of Langerhans from the pancreas are destroyed (Figure 2A). Hence, hyperglycemia develops; however, since insulin controls glucose intake, lack of insulin creates of state of cellular starvation. Both hyperglycemia and cellular starvation coexist and, under certain conditions such as T1D, can generate metabolic stress.

The metabolic stress will activate the HPA axis (Figure 2B) in an attempt to restore homoeostasis and recover from metabolic disturbance. The HPA axis activation will ultimately raise basal plasma GCs levels, which will be followed by the shut down of the HPA axis via GCs negative feedback.

However, previous reports in T1D animal models showed GR downregulation in the hippocampus (Tornello *et al*, 1981) and HPA axis hyperactivity (Chan *et al*, 2001 and 2002) (Figure 2C). These results explain, in part, the chronic hypercorticism observed in T1D patients and animals. On the other hand, GCs exert an hyperglycemic effect, inhibiting cellular glucose uptake in the periphery and also in neurons and astrocytes of brain regions such as the hippocampus (Munck *et al*, 1984; Sapolsky, 1992). Therefore, pre-existing hyperglycemia will become chronic state. In this way, continuous metabolic alterations will contribute to the defective shut-off of the stress response. When HPA axis activity is chronically elevated or managed inefficiently, various forms of pathophysiologies are promoted, such as dysfunction of the hippocampus (McEwen 1998; Sapolsky, 1992).

In summary, in agreement with published reports, diabetic animals showed a sustained stimulation of the HPA axis, with elevated basal plasma GC levels. In addition, adrenal hypertrophy, and spleen and thymus atrophy was found; characteristics also of long-lasting exposure to high amounts of GCs (Scribner *et al*, 1993; Oster *et al*, 1988; Bellush, *et al*, 1991). The observation that diabetic animals show a poor shut-off of the stress response (Magarinos and McEwen 2000; Chan *et al*, 2001; McEwen *et al*, 2002) suggests insensitivity to feedback mechanisms and is consistent with the reported resistance to the dexamethasone suppression test (Scribner *et al*, 1993) and downregulation of GR levels in the hippocampus of STZ rats (Tornello *et al*, 1981). In conclusion, processes to adapt to the metabolic imbalance will be created in T1D, involving poor glucose homeostasis, chronically elevated GC levels, increased HPA axis reactivity, and metabolic adjustments. Extreme metabolic adjustments can induce and significantly accelerate hippocampal remodeling, increasing its vulnerability to diabetes, and cognitive dysfunctions.

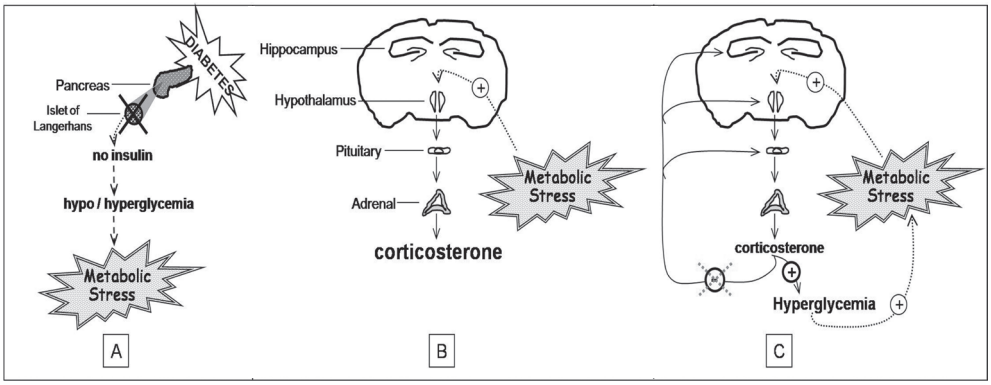


Figure 2. *HPA axis alterations in type 1 diabetes. Metabolic stress develops when insulin-producing cells destroy leads to hyperglycemia (A). As a consequence, the activation of the HPA axis triggers an increase in corticosteroids levels (B). The chronic high corticosterone concentration, on one hand exacerbates glucose secretion, and on the other hand acts on the HPA axis in order to shut-off the stress response and restore the homeostasis (C). According to the literature, in type 1 diabetes this response is disrupted.*

3. Impact of Type 1 Diabetes

3.1. Central Nervous System

Diabetic nephropathy, neuropathy, and retinopathy are traditionally considered the late complications of diabetes, whereas CNS was believed to be spared from diabetic complications. However, substantial evidence from clinical and experimental studies demonstrates that diabetes causes primary disease duration-related impairments in CNS function besides secondary sequelae of cerebrovascular events mediated by diabetic macrovascular disease (Li *et al*, 2002; Sima *et al*, 2004). In particular, hyper- and hypoglycaemic episodes may result in acute cerebral dysfunction (Biessels *et al*, 1994; Cryer *et al*, 1994). The consequences of these acute insults to the brain are well recognized. However, there is little knowledge about functional and structural cerebral alterations that develop more insidiously in diabetes. Long-term effects of diabetes on the brain are manifested at the structural, neurophysiological and neuropsychological level. The emerging view is that the diabetic brain features many signs that are best described as accelerated aging.

In diabetic rodents, structural abnormalities including synaptic and neuronal alterations, degeneration, neuronal loss, glycogen accumulation, dilated and fragmented endoplasmic reticulum, increased microtubuli, and irregular nuclei have been demonstrated (Bestetti and Rossi, 1980 and 1982; Garris *et al*, 1982, Luse, 1970, Mukai *et al*, 1980, Magarinos and McEwen 2000, Saravia *et al*, 2001; McEwen 2002). In addition, impaired long-term potentiation in the hippocampus (indicative of pre- and post-synaptic deficits) was reported (Biessels *et al*, 1996). The hippocampus is also vulnerable to damage by stroke and head trauma, susceptible to damage during aging, chronic stress (Sapolsky

1992), and sensitive to the effects of diabetes (Gispen and Biessels, 2000; Magarinos and McEwen 2000; Saravia *et al*, 2002). However, it is also a plastic and adaptable brain region that is capable of considerable structural reorganization. Studies from McEwen (2002) have shown that STZ induction perturbs structural plasticity of the hippocampus and its sensitivity to glucose and oxidative stress. His studies suggest that STZ-diabetes might cause a reduced number of dendrite spines and decreased total length of dendrites of pyramidal neurons of the hippocampus (Magarinos and McEwen, 1995). Other studies by Martinez-Tellez *et al* (2005) showed that dendritic morphological changes also occurs in pyramidal neurons located in structures related to cognitive processes such as, prefrontal cortex, occipital cortex and hippocampus, in the STZ-diabetic rats. The authors suggest that these results together with the available literature, indicate that nitric oxide (NO), GCs, stress, astrogliosis, and glutamate may participate in the dendritic morphological changes.

3.2. Behavior

Diabetes-related cognitive dysfunctions were first reported in 1922 (Miles and Root, 1922). Subsequent studies have demonstrated impairments in CNS function. Impairments in learning and memory, problem solving, and intellectual development have been documented in T1D patients (Ryan and Williams, 1993; Ryan *et al*, 1993; Kramer, 1998; Parisi and Uccioli, 2001; McCarthy *et al*, 2002; Schoenle, 2002). Cognitive dysfunction and impaired intellectual development are evident in a duration-related manner in T1D patients independent of hypoglycemic episodes (Kramer, 1998; Schoenle, 2002).

Stress and stress hormones affect different aspects of learning and memory. MR signaling can enhance performance on spatial hippocampal-dependent cognitive tasks (de Kloet *et al*, 1999) and its chronic blockade impairs spatial memory (Douma *et al*, 1998). Decreasing GR signaling attenuates the impairing effects of GR activation on cognition (Nicholas *et al*, 2006). Acute elevation of GCs facilitates the formation of memories of events associated with strong emotions (McGaugh, 2000; Roozendaal, 2000). Chronically, however elevated GC levels contribute to impairment of cognitive function and promote damage to brain structures such as the hippocampus (McEwen and Sapolsky, 1995; Lupien and McEwen, 1997; Sapolsky, 2002). This inverse-U function of GCs is a reflection of the diversity of receptors for GCs in the hippocampus (de kloet *et al*, 1993).

Impaired performances in the Morris water maze are typically observed in STZ-induced diabetes rats (Lowy *et al*, 1993; Biessels *et al*, 1996; Lupien and McEwen, 1997) and are associated with impaired LTP in the hippocampus (Biessels *et al*, 1996). It is reported that STZ diabetes does not disturb operant behaviors for food reward (Kaleeswari *et al*, 1986) but facilitates retention of passive avoidance in rats and mice (although not always) (Bellush and Rowland, 1989; Flood *et al*, 1990; Mayer *et al*, 1990). In addition, diabetic rodents consistently displayed performance deficits in more complex learning tasks, such as an active avoidance T-maze, or a Morris water maze

depending on the duration of STZ diabetes (Biessels *et al*, 1996 and 1998; Flood *et al*, 1990; Popovic *et al*, 2001). However, discrepancies exist among several behavioral studies and may be partially explained by differences in task complexity, animal models used and duration of diabetes. A key factor, however, appears to be the nature of the stimulus used in behavioral paradigms. There are clear indications that the physiological responses to a novel environment or to stressful stimuli, which are often part of learning paradigms, are larger in STZ-diabetic than in non-diabetic rodents (Bellush *et al*, 1991; Bellush and Rowland, 1989; Flood *et al*, 1990). For example, enhanced retention of simple passive avoidance task in diabetic rodents has been attributed to an increased sensitivity to the foot shock (Bellush and Rowland, 1989, Flood *et al*, 1990).

4. Scope of the thesis

4.1. Rational and Objective

Peripheral and autonomous neuropathies are well-known and devastating complications of type 1 diabetes. However, T1D can also impact the integrity of the CNS, and the reason why T1D affects CNS integrity remains to be elucidated.

Diabetic animals show high circulating glucocorticoid levels, increased sensitivity to stress, and morphological alteration in various brain areas. How these changes occur is not known, but hypercorticism *per se* can evoke a similar neurodegenerative cascade. The conditions of aberrant GCs levels appear (i) to enhance the vulnerability to metabolic insults of brain areas showing a high degree of plasticity, such as the hippocampus and (ii) may underlie the impairment of cognitive performance.

In T1D, a fundamental question in the central neuropathophysiology is whether GCs aggravate the functional and morphological signs of neurodegeneration and cognitive impairment. Therefore, the objective of the present study is to elucidate the role of GC excess and GC-stress system activity in T1D mice in relation to morphological indices for neuronal viability and cognitive performance.

4.2. Hypothesis

We hypothesize that under conditions of T1D, excess GCs and dysregulation of the GC-stress system will contribute to cerebral damage by making the brain more vulnerable to metabolic insults and causing concomitant cognitive disturbances. We propose that T1D leads to a more fragile state of the brain in which high levels of GCs may enhance the potential for damage and attenuate protective mechanisms, thus precipitating impairment in cognitive function. We expect to unravel GCs and diabetes interactions and relationships.

4.3. Questions to address

I. Is there neuronal damage in diabetes? (maybe as a consequence of GCs excess in diabetes?)
II. What is the pattern of HPA (re)activity in response to diabetes in time?
III. Do cognitive disturbances parallel these changes?
IV. Are GCs responsible for brain alterations in diabetes?
V. Will treatment with anti-glucocorticoids (RU486) prevent and/or restore these alterations?

4.4. Experimental approach

To allow generalization of the results, pharmacological and genetic animal models for diabetes, the STZ-induced and NOD mice, respectively, will be used. For both animal models there are indications of an aberrant functioning GC-stress system (i.e. GC hypersecretion). To test our hypothesis we planned:

I. To measure parameters of neuronal damage in NOD pre-diabetic, non-diabetic and diabetic mice and in STZ-diabetic and control mice.
II. To test HPA axis (re)activity in both models. For this purpose blood samples will be collected to measure basal concentrations of GCs and ACTH. Also central parameters of HPA activity (*in situ* hybridization of mRNA of MR, GR, CRH, AVP) will be measured.
III. To test cognitive abilities at certain time points of specific HPA (re)activity in the Morris water maze, novel-place recognition, open field, elevated plus maze, forced swimming test. General measures of activity will be recorded as well.
IV. To use the glucocorticoid antagonist RU486 in order to elucidate the role of GCs and attenuate vulnerability to damage and enhance protection. We discovered that RU486 acts like a double-edged sword: the antagonist blocks the damaging impact of excess GR stimulation while maintaining the beneficial effects of the 'neuroprotective' MR. After four consecutive days of RU486 administration, prevention and/or amelioration of neuropathological signs and restoration of cognitive abilities will be studied.

4.5. Chapters

Chapter 2 delineates the HPA axis functionality in a genetic model of T1D, the NOD mice. Central parameters of the HPA axis as well as C-peptide and cytokine levels were measured in pre-diabetes and diabetes states. In this model, the results suggest that an enhanced ACTH release may signal the onset of diabetes. **Chapter 3** addresses the underlying mechanism of hypercorticism in STZ mice. Central parameters of the HPA axis and specifically adrenal function were investigated at different time points after STZ-injection. The study demonstrates that adrenal hypersensitivity to ACTH precedes and

maintains the state of chronically elevated glucocorticoids levels. **Chapter 4** describes molecular parameters in the hippocampus of the STZ-diabetic mice that could reflect functional abnormalities of astrocytes and neurons. Parameters of astrocytic and neuronal disturbances such as apolipoprotein E and markers for oxidative stress and early gene expression respectively, were measured between control and diabetic mice. The results showed hippocampal disturbances in this model of T1D, which could be a primary basic mechanism underlying the well-known brain alterations associated with diabetes. **Chapter 5** reveals the role of GCs in STZ-induced diabetic mice, at the molecular and cognitive levels. After the administration of glucocorticoid receptor antagonist (RU486 or mifepristone) for 4 consecutive days to non-diabetic and diabetic mice, the novel placement recognition test was performed and molecular hippocampal parameters reflecting functional abnormalities were measured. Normalization of neuropathological signs and cognitive abilities was found. In **Chapter 6** the experimental data are discussed and placed in a conceptual framework highlighting the central action of glucocorticoids in the onset and progression of diabetes neuropathology.

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Chapter 2

Enhanced ACTH release precedes the onset of diabetes in the NOD mice ?

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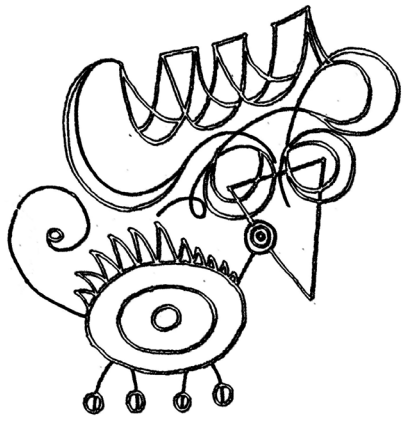

Abstract

Defects in the hypothalamus-pituitary-adrenal (HPA) axis regulation may play a role in further exacerbation of the autoimmune response against the pancreas beta-cells preceding the onset of diabetes. In a model of autoimmune type 1 diabetes, the nonobese diabetic (NOD) mouse, were reported: 1) increased levels of circulating glucocorticoids, suggesting activation of the HPA axis and 2) disturbances in brain areas regulating the HPA system, such as the hippocampus. Therefore, in the present study we hypothesized that altered HPA axis regulation in NOD mice may signal the onset and progression of the disease. Hence, we examined molecular markers of the hippocampus and the HPA axis in diabetic and non-diabetic littermates. The results revealed that a group of non-diabetic mice presents high ACTH release and low corticosterone levels suggesting adrenal hyporesponsiveness. This finding raises therefore the question whether it is possible that the profound ACTH activation precedes the onset of type 1 diabetes mellitus in a group of non-diabetic NOD mice. During full-blown diabetes glucocorticoids are elevated and ACTH is significantly lower as compared to non-diabetic littermates, suggesting a switch of hypo- to hyperresponsiveness of the adrenals to ACTH. Moreover, negative feedback regulation seems compromised because of downregulation of the glucocorticoids receptor in hippocampus and hypothalamus.

IN THE NONOBESE DIABETIC (NOD) mouse, a model of autoimmune type 1 diabetes (T1D), autoimmunity against beta cells is evident as early as 4 weeks of age, when infiltration of mononuclear cells into pancreatic islets (insulitis) is observed. The frequency of insulitis reaches 70% to 90% by 9 weeks of age, and by 20 weeks of age, almost 100% of mice of both sexes develop insulitis. Despite the development of insulitis in almost all NOD mice, only some of them develop overt diabetes: 80% to 90% of females and 10% to 50% of males. In this T1D model, brain alterations were described, such as: 1) increased number of glial fibrillary acidic protein reactive astrocytes in the hippocampus during the early post-weaning period (4 weeks of age, not yet diabetic animals), which was aggravated in diabetic mice (Saravia *et al*, 2002), 2) decreased cell proliferation after diabetes onset (Beauquis *et al*, 2007) and, 3) increased expression of hypothalamic arginine-vasopressin (AVP) and oxytocin proteins and mRNAs in diabetes (Saravia *et al*, 2001). Moreover, diabetic NOD mice also present increase levels of circulating glucocorticoids (Fiztpatrick *et al*, 1992. Amrani *et al*, 1994) suggesting activation of the hypothalamus pituitary adrenal (HPA) axis.

Animals at risk to develop endocrine/organ-specific autoimmune diseases show various pre-autoimmune aberrancies that need to interact abnormally before autoimmune disease can fully develop. In this abnormal interaction additional aberrancies in other regulatory systems may play a role in a further exacerbation of the self-directed immune response, such as defects in the HPA axis system (Lam-Tse *et al*, 2002).

This concept and the findings in pre-diabetic and diabetic states suggest that the NOD mouse is an excellent model to study the sequential changes in HPA axis activity. Therefore, in the present study the hypothesis was tested that altered HPA axis regulation may signals the onset and progression of the disease. Hence, we examined molecular markers of the hippocampal-HPA axis in NOD diabetic mice and NOD non-diabetic littermates. C-peptide concentrations were measured as an indicator for beta-cell activity, as well cytokines levels to monitor the autoimmune condition. The data suggest that in the pre-diabetic state a subgroup of animals can be identified which exhibit elevated ACTH and C-peptide levels.

Research Design and Methods

Animals

A NOD mouse colony was bred at the animal facility of the LACDR Leiden from adult NOD mice from Prof. Dr. Drexhage's laboratory (Department of Immunology, Erasmus University, Rotterdam, The Netherlands). Newborn pups were culled (4 males and 4 females per dam) and group housed (4 mice per cage). After weaning the rats were kept undisturbed under constant humidity (55±5 %) and temperature (23±2 ^{o}C) conditions with 12-12 light-dark hours cycle (lights on at 8 am). Food and water was provided *ad libitum*. The animal experiments were performed in accordance with the European

Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University.

Treatment

From week 12 of age glycemia levels were daily measured to assess diabetes (Accu-Chek Compact, Roche, Germany). Animals with blood glucose levels higher than 11mM were classified as overtly diabetic. At 25 days after diabetes onset male mice were decapitated between 9 am and 11 am. At the same time, randomly chosen non-diabetic littermates were sacrificed as control animals. After decapitation the brain was quickly removed, frozen in isopentane and stored at -80 ^{o}C until processing for later use in *in situ* hybridization. Trunk blood was collected for corticosterone, ACTH, C-peptide and cytokines (IL-1a, IL-6 and TNFα) measurements by radioimmunoassay (RIA) and Bio-Plex system respectively.

In situ hybridization

Determination of mRNA levels of MR, GR, AVP and CRH were measured on coronal brain cryosections (14 μm) containing hippocampus (distance from bregma -1.7 to -2.06 mm) and PVN (distance from bregma: -0.7 to -1.06 mm) (Paxinos and Frankling, 2001). Two or three sections from each mouse were mounted on poly-l-lysine (Sigma) coated slides and stored at −80 °C. *In situ* hybridization procedure follows the previously described by Revsin *et al* 2008. The mean of 4-6 measurements of each riboprobe were calculated for each animal.

Radio immuno assay (RIA)

Trunk blood was collected individually in labeled potassium-EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). Blood samples were kept on ice and later centrifuged for 15 minutes at 3000 rpm at 4 ^{o}C. Plasma was transferred to clean tubes and stored frozen at -20 ^{o}C until the determination of corticosterone, ACTH by MP Biomedical RIA kit (ICN, Biomedicals Inc., CA). C-peptide concentrations were determined with a RIA kit following the manufacturer's instructions (Linco Research St. Charles, Missouri, USA).

Cytokine determination

Cytokines were determined at the Luminex Core Facility (University Medical Center, Department of Pediatrics, Utrecht, The Netherlands) as previously described (de Jager *et al*, 2005). The Bio-Plex system employing the Luminex multi-analyte profiling technology (xMAP), allows individual and multiplex analysis of up to a hundred different mediators in a singe well containing a sample volume of 10 μl.

Data analysis and statistics

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Software (version 4). For corticosterone and ACTH plasma levels, 28 non-diabetic and 13 diabetic mice were used. For C-peptide and cytokines concentrations and *in situ* hybridizations 6 mice per group were randomly chosen from the pool of non-diabetic and diabetic mice; the mRNAs expression values were assessed by optical density (o.d.) of the signal on autoradiographic film. Statistical analysis was performed by one way ANOVA plus Bonferroni post test. Statistical differences were considered significant when $p<0.05$.

Results

Corticosterone, ACTH, C-peptide and pro-inflammatory cytokine concentrations.

In diabetic mice (glycemia: 21.72±3.61 mM, $p<0.05$ vs non-diabetic), basal corticosterone levels increased significantly compared to non-diabetic littermates (Figure 1A). While the corticosterone levels were elevated, basal plasma ACTH in the same animals was significantly decreased after diabetes onset as compared to non-diabetic mice (glycemia: 8.64 ±0.68 mM) (Figure 1B). However, a subgroup of non-diabetic animals (non-diabetic group 2) exhibits profoundly elevated ACTH levels. Based on this observation of different ACTH concentrations between the non-diabetic mice, 2 subgroups were identified: non-diabetic group 1 = non-diabetic mice with non altered ACTH (100.9± 25.17 pg/ml), and non-diabetic group 2 = non-diabetic mice with high ACTH (1934± 203.7 pg/ml) (>1000 pg/ml, $p<0.001$ vs non-diabetic group 1). These two subgroups were euglycemic and did not differ in body weight (data not shown).

C-peptide determination was performed to asses beta-cell function. Figure 1D shows that non-diabetic mice with highly elevated ACTH levels (non-diabetic group 2) also had significantly elevated C-peptide concentrations compared to non-diabetic group 1 and diabetic littermates. The C-peptide concentrations of non-diabetic group 1 mice were similar to non-fasting levels from control c57Bl/6 mice (0.5 to 1 nM, data not shown), indicating that the non-diabetic group 2 animals produce elevated C-peptide secretion. Additionally, C-peptide levels of diabetic mice are significantly decreased as compared to non-diabetic mice (group 1 and group 2).

Pro-inflammatory cytokines IL-1 alpha, IL-6 and TNF-alpha did not vary among the groups (data not shown).

HPA axis disturbances

In situ hybridization revealed no differences in the mRNAs expression of MR in the hippocampus and CRH in the PVN between diabetic and non-diabetic mice (data

not shown). However, GR mRNA in the hippocampus (CA1 and DG) and PVN was significantly decreased (Figure 2A and 2B), while AVP mRNA was significantly elevated in diabetic mice as compared to non-diabetic (Figure 2C).

The two groups of non-diabetic mice, which differed in ACTH and C-peptide levels, did not exhibit differences in expression of any of the above-mentioned mRNAs.

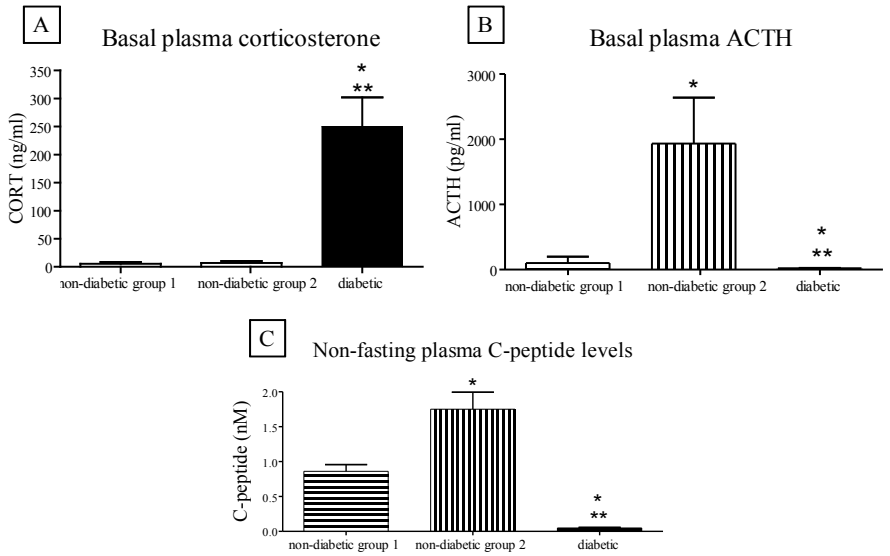

Figure 1. *Plasma levels of corticosterone, ACTH and C-peptide. A) Basal corticosterone (CORT) concentration in non-diabetic (group 1 and 2) and diabetic NOD mice. B) Basal ACTH concentration of non-diabetic (group 1 and 2), and diabetic NOD mice. C) Plasma C-peptide concentrations for non-diabetic (groups 1 and 2) and diabetic NOD mice. The data are expressed as mean±SEM. For CORT and ACTH measurements n= 28 non-diabetic mice (group 1 n=16, group 2 n= 12) and n= 13 diabetic mice. For C-peptide measurements n= 6 animals per group. *p<0.05 versus non-diabetic group 1 mice. **p<0.05 versus non-diabetic group 2 mice .*

Discussion

Our findings identify a subgroup of non-diabetic NOD mice showing elevated ACTH concentrations without the concomitant increase in corticosterone. Although the pre-diabetic phenotype seems to be characterized by adrenal hyporesponsiveness in view of high basal ACTH vs typical basal corticosterone levels, the opposite is observed in long-term diabetes. During full blown type 1 diabetes basal corticosterone level is elevated and basal plasma ACTH level is significantly lower as compared to non-

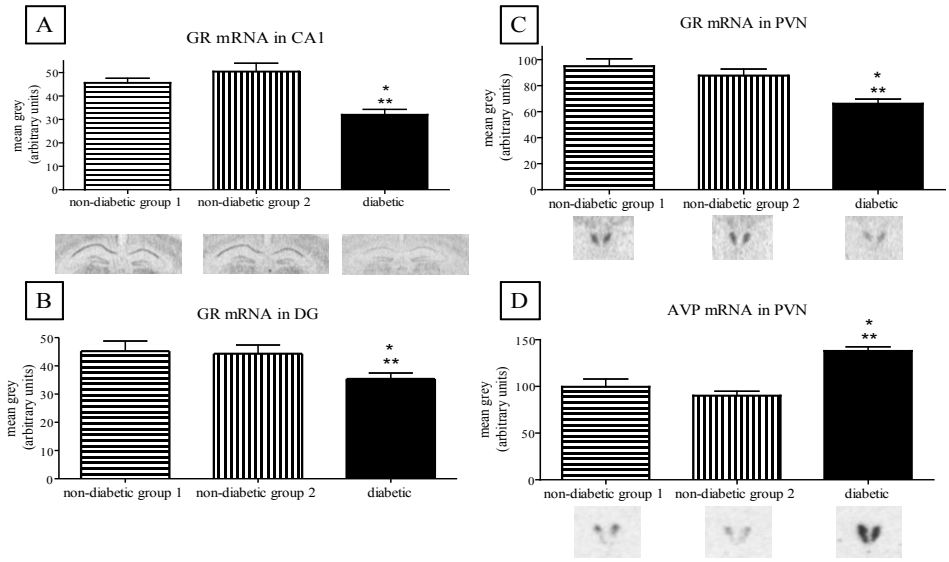

*Figure 2. In situ hybridizations for GR and AVP mRNAs. GR mRNA expression in CA1 (A) and dentate gyrus (DG) (B) areas of the hippocampus. mRNAs expression in the PVN of GR (C) and AVP (D). Microphotographs for hippocampal and PVN GR, and PVN AVP mRNAs are located below its respective group. Data is expressed as mean±SEM of the mean grey values (arbitrary units of the optical density). *p<0.05 versus non-diabetic group 1 mice. **p<0.05 versus non-diabetic group 2 mice . n= 6 per group.*

diabetic littermates, a condition suggesting hyperresponsiveness of the adrenals to ACTH. Moreover, downregulation of the glucocorticoid receptor in the hippocampus and PVN of diabetic animals suggests that the capacity of corticosterone to suppress the HPA axis is diminished. Such an impaired negative feedback would further promote hypercorticism.

The finding raises the question whether the activation of ACTH release precedes full-blown diabetes in the NOD mouse model of autoimmune type 1 diabetes. We showed in the present study, that enhanced ACTH release occurs concomitantly with increased C-peptide release (non-diabetic group 2), indicative of increased beta-cell activity. Mild transiently elevated insulin levels have been reported very early during the pre-diabetic period in NOD mice, developing between 2 and 4 weeks of age and persisting until 8 weeks of age (Amrani *et al*, 1998; Homo-Delarche, 1997; Orban *et al*, 2001). Moreover, transient hyperinsulinemia has also been observed in another model of spontaneous T1D, the BioBreeding (BB) rats, a few days before the onset of T1D, and perifused inflamed, but not uninflamed, islets exhibit beta-cell hyperactivity (Nakhooda *et al*, 1978; Teruya *et al*, 1993). In view of these and our results, it seems likely that the group of mice

with elevated C-peptide might develop T1D. Therefore, ACTH release may precede the cascade of endocrine events triggered by the destruction of insulin-producing cells. However, definite proof for these sequences of events requires longitudinal studies as well as the demonstration of more severe insulitis in these animals (non-diabetic group 2).

It is also noteworthy that in this subgroup in the face of ACTH hypersecretion, corticosterone levels remain unaffected, indicating decreased sensitivity of the adrenals to ACTH and/or impaired adrenal function. Such a relatively reduced adrenal function would facilitate the progression of autoimmunity, but how would such hyporresponsiveness develop? Lymphocytic infiltration of the adrenal gland was previously described in NOD mice. However, no signs of immune destruction of the adrenal neither in pre-diabetic nor in diabetic NOD mice were detected (Breidert *et al*, 1998). Moreover, lymphocytic infiltration of the adrenal glands was not accompanied by changes in corticosterone levels (Beales *et al*, 2002). From these studies, we can discard the idea that the lack of a corticosterone response is due to adrenal immune destruction in the subgroup of pre-diabetic NOD mice with high ACTH concentration. Although our results on plasma cytokine concentrations did not provide any difference between groups at the time of decapitation, we can not exclude changes in the adrenal cytokine balance to explain the lack of increased corticosterone concentrations in view of the increase in ACTH levels. Apparently, the adrenal glands were hyposensitive to ACTH and additional analysis of ACTH receptor expression levels, as well as the concentration of the various cytokines in the adrenal glands might help to elucidate this phenomenon.

In full-blown diabetes, we found, as previously described by Fitzpatrick *et al* in 1992, corticosterone hypersecretion indicating HPA axis activation. However, ACTH levels are significantly decreased in NOD diabetic mice, suggesting hyperresponsiveness of adrenals to ACTH. These data are in parallel with the ones described by our laboratory in the pharmacological model of T1D, the STZ-diabetic mice (Revsin *et al*, 2008). In the later model, we described a possible underlying mechanism for this observation: 11-days diabetic mice show upregulation of adrenocortical ACTH receptors (melanocortin 2 and 5) and increased corticosterone release from adrenocortical cell cultures challenged to ACTH.

In conclusion, the present data indicate that the profound ACTH activation is present in a group of non-diabetic NOD mice without increased corticosterone levels. It is conceivable that this state of adrenal hyporesponsiveness facilitates autoimmunity to the beta-cells; hence, increased ACTH release may precedes the onset of type 1 diabetes mellitus which in its full development switch to hyperresponsiveness of the adrenals and hypercorticism.

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CHAPTER 3

Adrenal Hypersensitivity Precedes Chronic Hypercorticism in Streptozotocin-Induced Diabetes Mice

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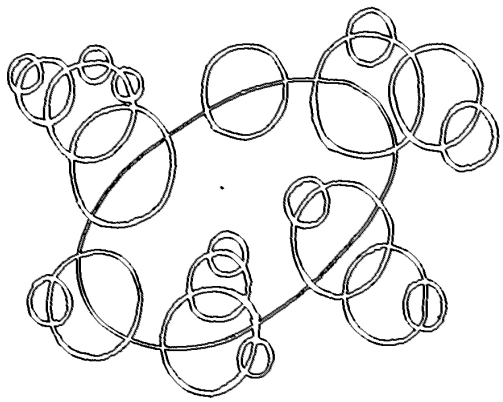

Abstract

Previous studies have demonstrated that type 1 diabetes is characterized by hypercorticism and lack of periodicity in adrenal hormone secretion. In the present study,wetested the hypothesis that hypercorticism is initiated by an enhanced release of ACTH leading subsequently to adrenocortical growth and increased output of adrenocortical hormones. To test this hypothesis,we used the streptozotocin (STZ)-induced diabetes mouse model and measured hypothalamic-pituitaryadrenal axis activity at different time points. The results showed that the expected rise in blood glucose levels induced by STZ treatment preceded the surge in corticosterone secretion, which took place 1 d after diabetes onset. Surprisingly, circulating ACTH levels were not increased and even below control levels until 1 d after diabetes onset and remained low until d 11 during hypercorticism. In response to ACTH (but not vasopressin), cultures of adrenal gland cells from 11-d diabetic mice secreted higher amounts of corticosterone than control cells. Real-time quantitative PCR revealed increased expression of melanocortin 2 and melanocortin 5 receptors in the adrenal glands at 2 and 11 d of STZ-induced diabetes. AVP mRNA expression in the paraventricular nucleus of the hypothalamus was increased, whereas hippocampal MR mRNA was decreased in 11-d diabetic animals. GR and CRH mRNAs remained unchanged in hippocampus and paraventricular nucleus of diabetic mice at all time points studied. These results suggest that sensitization of the adrenal glands to ACTH rather than an increase in circulating ACTH level is the primary event leading to hypercorticism in the STZ-induced diabetes mouse model.

TYPE 1 DIABETES (T1D) is a common metabolic disorder characterized by profound dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and disturbances in central nervous system functions (1–7). Among the alterations in the central nervous system functions, we previously reported enhanced expression of markers for astrogliosis and oxidative stress in the hippocampus of uncontrolled streptozotocin (STZ)-induced diabetic mice (8, 9). The hippocampus plays a crucial role in processes underlying learning and memory (10, 11) and has a transsynaptic neural input to CRH neurons in the paraventricular nucleus of the hypothalamus (PVN) (12). This hippocampal output to the PVN is known to inhibit HPA axis activity under basal and stressful conditions (13). Thus, it is conceivable that alterations in hippocampal markers could reflect disturbance in hippocampal output, which in turn could contribute to HPA axis dysregulation, leading to hypercorticism in the diabetic animals (14).

Previous reports indeed have shown alterations at various levels of the HPA axis such as increased hippocampal mineralocorticoid receptor (MR) and hypothalamic CRH mRNA and circulating ACTH levels (15). These alterations are believed to underlie corticosterone (B) hypersecretion in diabetes (16). At the time of full-blown diabetes, increase in central drive to the HPA axis at and/or above the level of the PVN has been reported (15, 16). This increased HPA axis drive would operate in the face of decreased B negative feedback sensitivity when diabetes is developed at d 8 after STZ treatment. However, the initial trigger of the sustained activation of the HPA axis is not known.

The current study was designed to assess some of the initial changes in the HPA axis at the onset of diabetes (author's operational definition of the first measured hyperglycemia after STZ injection) that eventually lead to chronic B hypersecretion in T1D. Based on previous studies, the hypothesis was tested that hypercorticism would start with enhanced release of ACTH leading subsequently to adrenocortical growth and stimulation of its adrenal melanocortin receptors 2 and 5 (MC2 and MC5). To test this hypothesis, we measured various key components of HPA axis activity at different time points after administration of STZ into c57BL/6 mice. These include plasma ACTH and B levels as well as CRH and vasopressin (AVP) mRNAs expression in the PVN. MR and glucocorticoid receptor (GR) expression were measured in the hippocampus and PVN. Moreover, the expression of AVP 1a receptor (V1aR), MC2 receptor (MC2R), and MC5R were measured in the adrenals, whereas their ability to secrete B was tested with ACTH and AVP stimulation using adrenal cell culture.

We find that the initial hypersecretion of B at the onset of diabetes occurs as a result of adrenal gland hypersensitization to ACTH rather than being triggered by elevated ACTH levels.

Materials and Methods

Animals

Twelve-week-old c57BL/6 male mice (Janvier, Le Genest Saint Isle, France) were group

housed (two or three mice per cage, randomly mixing vehicle- or STZ-injected animals) under constant humidity (55 ± 5%) and temperature (23 ± 2 ºC) conditions, with 12-h light, 12-h dark cycle (lights on at 0800 h) at the animal facility of the LACDR, Leiden. Food and water was provided *ad libitum*. The animal experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University (UDEC No. 04096).

Treatment

Mice received a single ip dose of 170 mg/kg STZ (Sigma Chemical Co., St. Louis, MO) at 0900 h in 0.5 M sodium citrate buffer (pH 4.5) or vehicle; 48 h after injection, diabetes was assessed by glucose levels in blood in the nonfasting condition (Accu-Chek Compact; Roche Diagnostics, Mannheim, Germany). Plasma glucose level measurements and killing of mice were performed between 1000 and 1200 h for all the experiments, except for the time course studies of Fig. 2. Animals with glucose levels higher than 11 mM were classified as overtly diabetic.

Experiments

Two and 11 d after diabetes onset (acute and chronic diabetes, respectively), animals were decapitated; brain and adrenal glands were quickly removed, frozen in isopentane, and stored at -80 ºC until processing for later use in *in situ* hybridization and real-time quantitative PCR (RT-qPCR) procedures, respectively. For adrenal cell cultures, adrenal glands were immediately processed for direct use, and trunk blood was collected for RIA measurements.

In situ hybridization

Determination of mRNA levels of MR, GR, AVP, and CRH were measured on coronal brain cryosections (14 μm) containing hippocampus (distance from bregma, 1.7 to 2.06 mm) and PVN (distance from bregma, 0.7 to 1.06 mm) (17). Two or three sections from each mouse were mounted on slides coated with poly-l-lysine (Sigma) and stored at -80 ºC. The sections were fixed for 30 min in freshly made 4% paraformaldehyde (Sigma) in PBS (pH 7.4), rinsed twice in PBS, acetylated in triethanolamine (0.1 M, pH 8.0) with 0.25% acetic anhydride for 10 min, rinsed for 10 min in 2 standard saline citrate (SSC: 150 mM sodium chloride, 15 mM sodium citrate), dehydrated in an ethanol series, air dried, and stored at room temperature until the *in situ* hybridization. The cRNA probes for GR and MR (mouse, exon 2 coding region) (18, 19), CRH (rat, full-length coding region) (20), and AVP (rat, exon C coding region) (21) were used. The antisense cRNA probes were transcribed from a linearized plasmid. *In situ* hybridization was performed using labeled ribonucleotide probes (labeling reaction: 10% 10 transcription buffer, 20%

nucleotide mix (33.3% 10 nM ATP plus 33.3% 10 nM CTP plus 33.3% 10 nM GTP), 12% 100 mM UTP, 4% ribonuclease (RNase) inhibitor, 5% riboprobe, 19% ddH2O, 25% [35S]UTP, 5% polymerase), reaching 80–90% transcription efficiency. A 100-µl aliquot of hybridization mix [50% formamide, 20% dextran sulfate, 1.2 mM EDTA (pH 8.0), 25 mM sodium phosphate (pH 7.0), 350 mM sodium chloride, 100 mM dithiothreitol and 1% Denhardt's, 2% RNA-DNA mix (50% t-RNA plus 50% herring sperm DNA), 0.2% nathiosulfate and 0.2% sodium dodecyl sulfate] containing $2x10^6$ dpm from each riboprobe was added to each slide. Coverslips were put on the slides, which were hybridized overnight in a moist chamber at 55 °C. The next morning, coverslips were removed and the sections washed in graded salt solutions at optimized temperature [10 min 2% SSC at 55 °C, 15 min RNase A solution (0.2% RNase A plus 10% 5 m NaCl plus 1% 1 M Tris-HCl plus 88.8% dH2O) at 37 °C, two times for 10 min each 2% SSC at 65 °C, 15 min 2% SSC/formamide at 65 °C, 10 min 1% SSC at 65 °C, and 10 min 0.1% SSC at 65 °C]. After the washing steps, sections were dehydrated in a series of ethanol baths and air dried. The signal was quantified from film Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and relative expression of MR, GR, CRH, and AVP mRNA was determined by computer-assisted optical densitometry (analysis 3.1; Soft Imaging System GmbH). The mean of four to six measurements of each riboprobe was calculated for each animal.

RIA

Trunk blood was collected individually in fasting (1700 and 2000 h) and nonfasting (0900 and 1300 h) conditions in labeled potassium-EDTA-coated tubes (1.6 mg EDTA/ml blood; Sarstedt AG & Co., Numbrecht, Germany). Blood samples were kept on ice and later centrifuged for 15 min at 3000 rpm at 4 °C. Plasma was transferred to clean tubes and stored frozen at -20 °C until the determination of ACTH and B by the MP Biomedical RIA kit (ICN Biomedicals Inc., Costa Mesa, CA). Insulin concentrations were measured with a RIA kit following the manufacturer's instructions (Linco Research, St. Charles, MO).

RT-qPCR

Total RNA was extracted from the adrenals using TRIzol RNA isolation reagent (Invitrogen) according to the manufacturer's recommendations. After isolation, total RNA was purified using the QIAGEN RNEasy Mini Kit RNA Cleanup (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. RNA quality was assessed with the Nanodrop (Isogen Life Science, Maarsen, The Netherlands). Before cDNA synthesis, all RNA samples were treated with deoxyribonuclease I (Invitrogen Life Technologies), according to the manufacturer's protocol. Synthesis of cDNA was performed in a total volume of 20 µl, using SuperScript II reverse transcriptase (Invitrogen Life Technologies). Each experimental sample of RNA (10 ng/µl) was

placed into the cDNA-synthesis reaction. Standard curves were generated by performing cDNA-synthesis reactions on 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.78 ng/µl input RNA. As a control for genomic contamination, RT samples were used. Primers for ACTH receptors, MC2R (forward 5'-AAATGATTCTGCTGCTTCCAA-3' and reverse 5'-TGGTGTTTGCCGTTGACTTA-3'), MC5R (forward 5'-TGGAACCCGTGAAGAATCAT-3' and reverse 5'- TCCTAAAATGCCATCCTCTGA-3'),and V1aR (forward 5'-GCCTACATCCTCTGCTGGAC-3' and reverse 5'-AGCTGTTCAAGGAAGCCAGT-3') were designed using the Ensemble database, Primer3, and the NCBI database BLAST, all accessible on the internet. The amount of the target genes was determined relative to the housekeeping gene 18S (forward 5'-GTAACCCGTTGAACCCCATT-3' and reverse 5'-CCATCCAATCGGTAGTAGCG-3') (22). Specificity of the primer sets was assessed with a cDNA sample (12.5 ng/µl) and a negative, RT^-, sample using the LightCycler and Light-Cycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). Dissociation curves were examined for each primer pair and controlled for specificity of the reaction and genomic contamination by checking the RT and no-template control samples. Then, for each primer pair, the standard curve (50, 25, 12.5, 6.25, 3.125, 1.562, and 0.78 ng/µl) was plotted, and the PCR efficiency was estimated. All used primer pairs displayed reaction efficiencies between 80 and 100%. Target gene cycle threshold values ranged from 18–32, whereas RT^- and no-template control samples showed no products after 40 cycles. PCR amplification of the cDNA was performed in a 20-µl reaction, using the LightCycler and LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit. A PCR MasterMix was made consisting of 7 µl PCR-graded water, 4 µl reaction mix, 4 µl PCR primers, and 5 µl cDNA. The Light-Cycler protocol started with a preincubation, heating for 10 min at 95 °C, followed by 45 cycles of 10 sec at 95 °C, 10 sec at 60 °C, and 10 sec at 72 °C for elongation. After the amplification, the program continued with a melting curve consisting of 15 sec at 65 °C after which the temperature was held at 4 °C.

Adrenocortical cell culturing

Immediately after decapitation, the adrenals were removed, their fat was cleaned, their individual weight was determined, and they were stored in 0.9% NaCl. Cell suspension was made by cutting the tissue into small pieces and placing in 2 mg crude collagenase (Sigma-Aldrich Inc., Steinheim, Germany) and 0.4% BSA in 1 ml DMEM buffer (25 mm HEPES, 4500 mg/liter glucose; BioWhittaker, Cambrex BioScience, Verviers, Belgium). The cell suspension was disrupted continuously by pipetting every 15 min during the 2-h incubation at 37 °C (atmosphere of 95% O_2 and 5% CO_2). The cell samples were then centrifuged twice at 100 xg (4 °C) for 10 min, and the cells were resuspended in 1.12 mg $CaCl_2$ in 1ml DMEM solution ($CaCl_2$ solution) and 0.4% BSA in DMEM. For testing the cell viability, cell suspensions were concentrated in the $CaCl_2$ solution and mixed with an equal volume of trypan blue (1 mg/ml in 0.9% NaCl), and cells containing liquid droplets and therefore excluding the dye (adrenocortical cells) were

counted under the microscope. The volumes of the cell suspensions were adjusted to 10,000 adrenocortical cells/ml with 5% BSA in DMEM and distributed into Eppendorf tubes, each consisting of 0.9 ml cell suspension, to be incubated for 60 min at 37 °C (at the described atmosphere).

The ACTH and AVP challenges were performed with $3.4x10^{-9}$ M $ACTH_{1–24}$ (Synacthen; Novartis Pharma BV, Arnhem, The Netherlands), 10^{-6} M AVP ($[Arg8]_{1–8}$; Organon, Oss, The Netherlands) or 5% BSA in DMEM (nonchallenged is negative control of the experiment). After cell suspension incubation, 0.1 ml of each concentration was added, and the samples were incubated for 2 h at 37 °C followed by centrifugation at 2500xg for 10 min (4 °C). The supernatant was collected and stored at -20 °C until later use for B determination by RIA.

Data analysis and statistics

All data are expressed as mean±SEM. Statistical analysis was performed using GraphPad Software (version 4). For pathophysiological measurements, six to seven mice per group were used and unpaired t test was applied. For *in situ* hybridization, five to eight mice per group were used, the values were assessed by OD of the signal on autoradiographic film, and the statistical analysis was by nonparametric two tailed Mann-Whitney *U* test or two-way ANOVA plus Bonferroni post test. For RT-qPCR, five mice per group and two-way ANOVA plus Bonferroni post test was used. For adrenal cell cultures, six to seven mice per group were employed, and two-way ANOVA plus Bonferroni post test was applied. Statistical differences were considered significant when $P < 0.05$.

Results

Pathophysiology

The pathophysiology of the diabetic mice resembled the characteristic clinical features of the disease. Table 1 shows at d 2 and 11 after diabetes onset, increased glucose levels (11 mM) and adrenal/body weight ratio, whereas body weight and thymus/body weight ratio were decreased. Absolute adrenal weights were similar at early time points after injection (4 h diabetic 1.77±0.06, control 1.93±0.04; 8 h diabetic 1.97±0.06, control 2.06 ±0.12; 24 h diabetic 1.87±0.08, control 1.68±0.06; 48 h diabetic 2.07±0.09, control 2.00 ±0.15 mg), and in 2-d diabetic mice and controls (diabetic 1.75±0.10, controls 1.52±0.07, mg). At 11 d after diabetes onset, adrenal weights were significantly higher (diabetic 2.83 ±0.12, control 1.74±0.16 mg, $P < 0.001$). Absolute thymus weight did not differ at the early time points after injection (4 h diabetic 23.18±1.43, control 22.20±1.51; 8 h diabetic 21.77±2.0, control 20.62±2.70; 24 h diabetic 17.95±1.7, control 21.50±1.30; 48 h diabetic 17.16±1.37, control 23.72±0.93 mg), however, it was significantly decreased in diabetic compared with control mice at d 2 (diabetic 6.39±0.6, control 24.83±1.97, P

< 0.0001) and d 11 (diabetic 10.13±1.29, control 29.32±4.21 mg, P < 0.01) after diabetes onset. In addition, diabetic mice exhibited increased food and water intake from diabetes onset (data not shown).

Plasma glucose, insulin, B, and ACTH

In diabetic mice, basal B levels increased significantly 1 d after diabetes onset, when circulating glucose levels rose above 11 mM (Fig. 1A) and were maintained at a high level until animals were killed. Control animals showed low basal B levels (10 ng/ml) (Fig. 1B). Because diabetic mice have lost circadian rhythmicity in HPA axis activity, this accounts for variations in the basal B levels from different sets of animals observed at 48 h after STZ injection in Fig. 1, A and B. Although B levels were elevated, basal plasma ACTH in the same animals was significantly decreased after diabetes onset compared with controls (acute diabetic 69.97±12.64, control 159.3±27.54; chronic diabetic 70.94± 8.03, control 158.4±20.31 ng/ml) (Fig. 1C). Surprisingly, the rise in B was not preceded by an increase in ACTH level in diabetic mice. A time course curve of ACTH at 4, 8, 24, 48, 59, 72, or 83 h after STZ injection (0900 h) showed no increase in ACTH at any time point compared with controls (Fig. 2A). At 4 h after injection, STZ-injected mice exhibited increased B concentration compared with vehicle-injected animals (Fig. 2B). However, in STZ-injected mice, B was comparable to control levels at 8, 24, 48, and 59 h after injection and commenced to increase from 72 h, i.e. at 1 d after diabetes onset (Fig. 2B). To evaluate the metabolic implications after STZ injection, insulin concentrations were also measured in the same animals. Figure 2B shows the time course of insulin levels and reveals that circulating insulin levels significantly increase 8 h after STZ injection. This increase is followed by significant decrease from 48 h onwards (Fig. 2C).

TABLE 1. Pathophysiological measures of diabetic mice at 2 or 11 d after diabetes onset

	Glycemia (mM)	δ Body weight (g)	Adrenal/body weight ratio	Thymus/body weight ratio
2 d after diabetes onset				
Controls	9.29±0.53	3.33±0.71	99.54±4.43	1022±91.84
Diabetic	25.90±1.19[c]	1.0±0.52[a]	158.9±15.91[b]	351.5±35.33[c]
11 d after diabetes onset				
Controls	9.643±0.52	2.857±0.51	66.95±6.17	1129±163.20
Diabetic	25.40±2.07[c]	-3.833±1.01[c]	160.40±10.94[c]	580.50±90.65[a]

Glycemia, difference (δ in grams) in body weight at the time of vehicle or STZ injections and at time of euthanasia, and adrenal/body weight and thymus/body weight ratios were assessed. Values are expressed as mean ± SEM; n = 6–7. Adrenal and thymus/body weight ratios are expressed as absolute weight x 1000 (g)/ body weight (g). [a]*P < 0.05 vs. control.* [b]*P < 0.01 vs. control.* [c]*P < 0.0001 vs. control.*

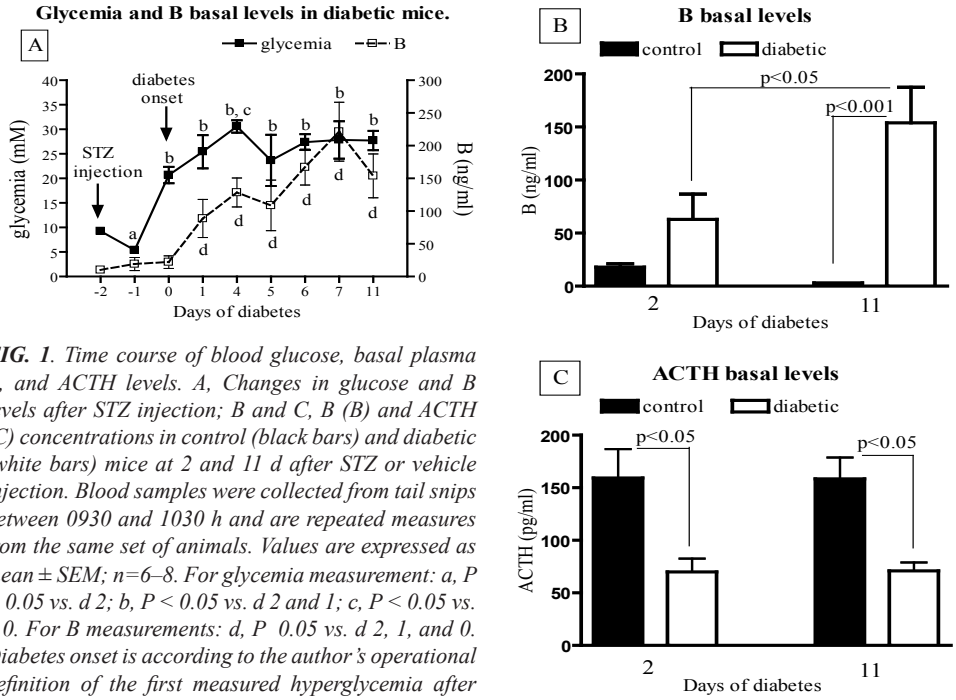

FIG. 1. *Time course of blood glucose, basal plasma B, and ACTH levels. A, Changes in glucose and B levels after STZ injection; B and C, B (B) and ACTH (C) concentrations in control (black bars) and diabetic (white bars) mice at 2 and 11 d after STZ or vehicle injection. Blood samples were collected from tail snips between 0930 and 1030 h and are repeated measures from the same set of animals. Values are expressed as mean ± SEM; n=6–8. For glycemia measurement: a, P < 0.05 vs. d 2; b, P < 0.05 vs. d 2 and 1; c, P < 0.05 vs. d 0. For B measurements: d, P 0.05 vs. d 2, 1, and 0. Diabetes onset is according to the author's operational definition of the first measured hyperglycemia after STZ injection.*

Adrenal regulation

ACTH and AVP challenges in chronic diabetes

Adrenocortical cells (10,000 cells per animal) were incubated with $3.4x10^{-9}$M $ACTH_{1–24}$, 10^{-6}M $AVP_{1–8}$, or 5% BSA in DMEM buffer as a negative control (nonchallenged). The ACTH concentration used for the challenge, which triggers B secretion in these cultures, was established by a dose-response curve to $1.7x10^{-11}$, $3.4x10^{-11}$, and $3.4x10^{-9}$M $ACTH_{1–24}$. In agreement with a previous report by Oitzl *et al*. (23), only the $3.4x10^{-9}$M dose triggers B release from control cell cultures, whereas the others do not have any effect. Figure 3A describes the response of the adrenal cells to $3.4x10^{-9}$M $ACTH_{1–24}$. Cultures of adrenal cells from diabetic mice secreted extremely high levels of B compared with cultures from control animals. Parallel cultures from the same mice challenged with 10^{-6}M $AVP_{1–8}$ showed different results. $AVP_{1–8}$ triggered B secretion in adrenal cell culture from control but not from diabetic animals (Fig. 3B).

RT-qPCR ACTH receptor and V1aR mRNA

The cDNA levels of the targeted genes were normalized with 18S cDNA expression.

Adrenal ACTH receptor MC2 mRNA expression was significantly increased in acute and chronic STZ-diabetic mice ($P < 0.05$) (Fig. 4A), and MC5R mRNA was significantly increased in chronic diabetes (P 0.01) (Fig. 4B). V1aR expression was not changed in diabetes (data not shown).

HPA axis disturbances

The *in situ* hybridization revealed no differences in hippocampal MR and GR mRNA expression between control and diabetic mice 2 d after the onset of the disease (Table 2). Chronic diabetic mice showed decreased MR mRNA in the hippocampus, which was significantly different only in the granular cells of the *dentate gyrus* (Table 2). The variation in mRNA expression between controls at 2 and 11 d (Table 2) represent the outcome of different experiments performed at different times in which the films were not adjusted against a standard. For that reason, the OD is expressed as arbitrary units from diabetic mice compared with control for each day. It is also noteworthy that significant MR mRNA up-regulation was found in the CA1 area of the hippocampus only on the day of diabetes onset (controls 20.43±3.73, diabetics 34.80±3.11 OD, arbitrary units; $P < 0.05$; $n = 5$).

In the PVN, the levels of AVP mRNA were significantly elevated in acute and chronic diabetic mice compared with controls (Table 2); CRH and GR mRNA expression did not differ between STZ-diabetic and vehicle-treated mice in acute and chronic diabetes (Table 2).

Discussion

The present study shows that the rise in blood glucose levels induced by STZ treatment precedes the surge in B secretion in the STZ-induced diabetes mouse model. This increase in circulating B levels seems to be triggered by a rapidly enhanced sensitivity of the adrenals to ACTH. Hence, the sustained hypercorticism in the face of a dramatically enhanced adrenal sensitivity to ACTH seems already established very early in the onset of diabetes. Therefore, the current data reject the hypothesis that hypercorticism would start with enhanced release of ACTH. This claim is further supported by the fact that there were actually no ACTH increases measured at any time points studied immediately after STZ injection and neither during the first day after diabetes onset nor at later time points when diabetes is fully established (i.e. after 11 d of diabetes). At all time points, ACTH concentrations remained below control levels.

The enhanced adrenal sensitivity to ACTH becomes apparent already from the increased adrenal weight and is demonstrated in vitro in cultures derived from adrenal cells taken from diabetic mice. Cultures of the adrenal gland cells from diabetic mice challenged with ACTH result in B hypersecretion. We then hypothesized that besides adrenocortical growth, stimulation of adrenal MC2R and MC5R might be a possible mechanism by which low concentrations of ACTH could maintain B hypersecretion.

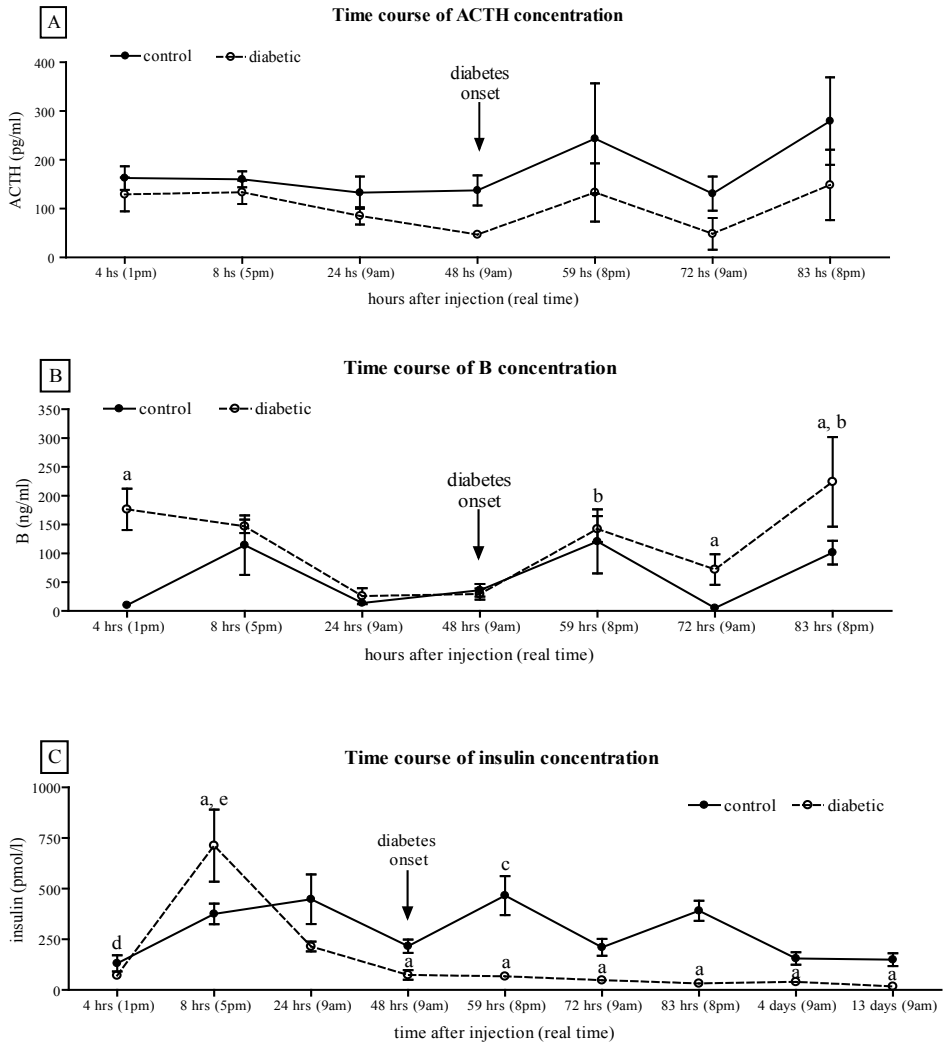

FIG. 2. *Time course of plasma ACTH, B, and insulin concentrations. A, ACTH levels; B, B levels; C, insulin levels after 4 (1300 h), 8 (1700 h), 24 (0900 h), 48 (0900 h), 59 (2000 h), 72 (0900 h), and 83 (2000 h) hours after STZ or vehicle injection. Values expressed mean ± SEM; n = 4–6. a, P < 0.05 vs. control; b, P < 0.05 vs. diabetic at 48 h; c, P < 0.05 vs. 4, 48, and 72 h and 4 and 13 d; d, P < 0.05 vs. 83 h; e, P < 0.05 vs. 4, 24, 48, 59, 72, and 83 h and 4 and 13 d. Four and 13 d after STZ injection is the same as 2 and 11 d of diabetes, respectively. Diabetes onset is according to author's operational definition of the first measured hyperglycemia after streptozotocin injection.*

Indeed, we found that ACTH receptors in the adrenals were up-regulated at 2 and 11 d of diabetes. The finding supports the hypothesis that a rapidly enhanced adrenal sensitivity to ACTH facilitates adaptation to the metabolic state induced by STZ in this diabetic model.

Other mechanisms inducing a rapid change in adrenal sensitivity could also be implicated. These mechanisms include splanchnic nerve input because splanchnic nerve stimulation was found to enhance the secretion of glucocorticoids in response to ACTH (24 –28). Sympathetic innervation and thus catecholamines from the medulla may also participate as an ACTH-independent input to adrenocortical function (29 –31). Adrenals also show a gated sensitivity to ACTH that is maintained in the absence of external signals but depends on the presence of a functional adrenal clockwork, which exerts its control on corticosteroid production and explains the circadian changes in adrenal sensitivity. In view of these findings, it has been suggested that light may directly entrain the adrenal clock via an autonomic input, thereby influencing circadian and possibly ultradian rhythms in B secretion (32, 33).

Other factors may be involved as well: 1) AVP exerts a direct stimulatory action on adrenocortical cells mediated through activation of typical V1aR. Our data demonstrate, however, that AVP does not mediate the hypercorticism in diabetes, because neither the B secretion from adrenocortical cultures nor adrenal V1aR expression was modulated compared with controls. 2) CRH/ACTH intraadrenal system can regulate adrenal steroidogenesis (29); a direct effect of CRH on adrenocortical steroidogenesis seems unlikely, because CRH had no effect on either isolated, dispersed adrenocortical cells (34) or on adrenocortical autotransplants deprived of chromaffin tissue.

An altered hippocampal input to the HPA axis was previously described in the STZ animal model of T1D (15, 35–38). However, discrepancies and similarities have been found in different models of T1D. Discrepancies are related to the HPA axis regulation. Chan *et al*. (15) reported a profound activation of the HPA axis in the rat model of STZ-induced diabetes, which was characterized by a marked increase in ACTH and B levels at 8 d after STZ injection. They found that the expression of AVP and CRH mRNAs in the hypothalamus and MR mRNA in the hippocampus was enhanced.Therefore, the authors suggested an increase in the central drive to the HPA axis that overrides the inhibitory influence of negative B feedback. Central to this reasoning was the up-regulation of MR in hippocampus, which is thought to modulate the inhibitory tone on HPA axis activity (39, 40).

In the present study, we found significant MR mRNA up-regulation in the CA1 area of the hippocampus only on the day of diabetes onset. However, at 11 d of diabetes, MR mRNA was significantly down-regulated in the hippocampus, which suggests that the inhibitory regulation on the HPA axis might be disrupted. This disruption could impair the shut-off response contributing to the observed chronic hypercorticism and could imply a time-dependent adaptation to the new metabolic condition. The nuclear MR in hippocampal neurons has a very high affinity to B and aldosterone, suggesting that this receptor is always extensively occupied. The MR signal is changed by altered receptor

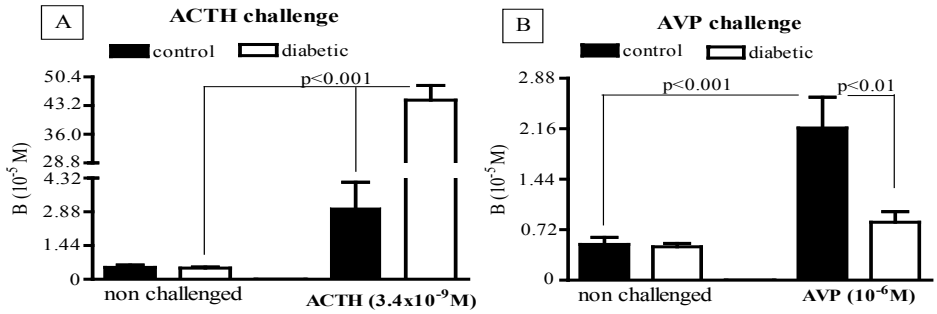

FIG. 3. *Corticosterone secretion from adrenal cell cultures (10,000 cells/ml) after 60 min incubation with 3.4x10⁹ M of ACTH1–24 (A) or 10⁶M of AVP1–8 (B) from 11-d diabetic mice. Black bars represent cultured cells from control animals and white bars from chronic diabetic ones.Values are expressed mean ± SEM; n=6–7. Nonchallenged indicates cultures from control and diabetic mice in which no ACTH or AVP were added.*

activity rather than ligand concentration. Hence, changes in nerve input have a profound influence on MR capacity (41). There is also a recently discovered membrane variant of the MR in the hippocampus, which may contribute to HPA axis regulation (42). Despite all these facts, we cannot offer an explanation why our results differ from those obtained by Chan *et al*. (15, 16).

Additional studies by Chan *et al*. (16) showed that adrenal sensitivity is not increased in uncontrolled STZ-diabetic animals as described before (37). They did not find a significant rise in B levels compared with controls after a low-dose ACTH stimulation test. Our data resemble, however, a study by Dallman *et al*. (43) showing that in response to food deprivation, elevated B levels occurred independent of an ACTH surge, suggesting an acute rise in adrenal sensitivity as the most proximal event. Another aspect of the study that remains unresolved is the question of how the thymus rapidly involutes in the face of moderately increased B levels in the STZ-treated animals.

Similarities in different models of the disease are also described in the literature: 1) decreased body weight gain and plasma insulin levels and increased food intake, water intake, plasma glucose, and B concentrations were reported in STZ-diabetic mice (8, 9 44) and rats (1, 7, 45), spontaneous T1D models such as the nonobese diabetic (NOD) mice (8, 46) and biobreeding rats (47, 48) and humans (49); 2) hippocampal alterations involving astrogliosis and decreased cell proliferation in STZ and NOD mice (8, 9, 44, 46) and STZ rats (50, 51); and 3) cognitive impairments in STZ-mice (Revsin, Y., N. V. Rekers, M. C. Louwe, F. E. Saravia, A. F. De Nicola, E. R. de Kloet, and M. S. Oitzl , submitted for publication), STZ rats (52), biobreeding rats (47), and humans (49). These similarities suggest no species differences and allow a generalization of the data we found in the STZ-diabetic mouse model.

The discrepancies between the present study and that of Chan *et al*. (15) could be explained by differences in the animal model used. First, although STZ-diabetic rats can survive up to 8 months (53), STZ-diabetic mice die a few months after onset of the disease (54, 55). Therefore, disparities in the severity of diabetes and routes of

STZ administration and dosages (due to species variation in response to the drug) can contribute to the observed differences. In this regard, whereas a single STZ injection of 65 mg/kg body weight ip induces diabetes in rats, it does not induce the disease in mice, showing that mice are more resistant to the action of STZ on the destruction of the pancreatic β-cells. In our hands, we found that 170 mg/kg body weight ip is the minimum dose that induces diabetes in the c57BL/6 mice with the same diabetic parameters described in the literature indicative of T1D (blood glucose, insulin levels, and weight gain) (56, 57). Second, in the studies by Chan *et al*. (15, 16), animals treated with STZ were given 10% sucrose in drinking water for the first 24 h after the STZ injection to prevent hypoglycemia. It is noteworthy that this STZ model of T1D features moderate diabetes with hyperglycemia and moderately reduced fasting insulin levels. In our model, no sucrose was administered and hypoglycemia was observed 24 h after the STZ injection. Then from 48 h after STZ injection, the animals become hyperglycemic (Fig. 1A) with low insulin levels at fasting (1700 and 2000 h) and nonfasting (0900 and 1300 h) (Fig. 2C).

Besides the temporal changes in glucose and insulin levels close to STZ injection, B concentration increases 4 h after STZ injection and later on from 72 h, which corresponds to 24 h after the onset of hyperglycemia (Figs. 1A and 2B). It is known that this drug has toxic effects in the first hours after its administration (58); hence, the B hypersecretion 4 h after STZ administration might be due to the STZ cytotoxicity *per se*. The increased B concentration, before β-cell destruction (insulin increased at 8 h after STZ injection), followed by its decrease to control levels supports our assumption. Therefore, the later B hypersecretion 3 d after STZ injection might be the result of the rise in blood glucose levels (or its consequences). However, one cannot discard the possibility that the transient hypoglycemia 24 h after STZ injection may also be relevant for the later hypercorticism. Future studies in our model on sucrose administration after STZ injection (15, 16) or insulin replacement (59) will help to fully understand the observed differences between these two STZ models.

In the present study, we showed HPA axis modulation in STZ-diabetic mice at 2

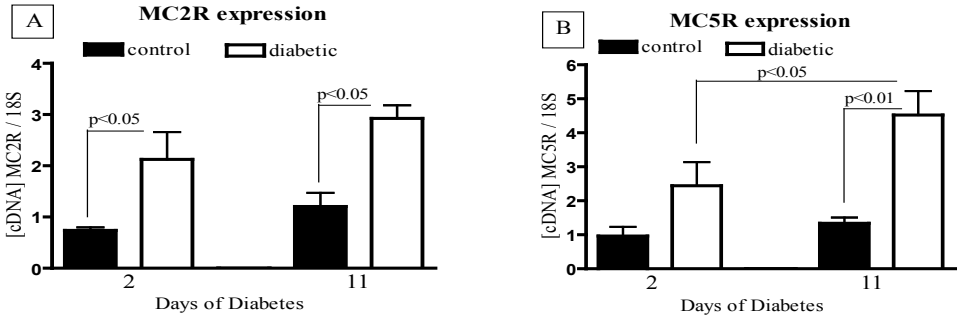

FIG. 4. *ACTH (MC2 and MC5) receptor mRNA expression in the adrenal glands. Adrenal MC2R and MC5R mRNAs were measured with RT-qPCR, in control (black bars) and diabetic (white bars) animals at 2 and 11 d after STZ or vehicle injection. Columns represent mean ± SEM; n = 5.*

TABLE 2. *Hippocampal MR and GR and hypothalamic AVP, GR, and CRH mRNA expression at 2 and 11 d after diabetes onset*

	Hippocampus			PVN		
	MR mRNA in DG	GR mRNA		AVP mRNA	GR mRNA	CRH mRNA
		CA1	DG			
2 d after diabetes onset						
Controls	40.65±10.82	41.58 ±4.33	34.32 ±1	50.79±3.63	54.68±13	109.7 ±10
Diabetic	35.84±4.20	42.66±6.35	31.70 ± 4.34	66.73±3.31[a]	78.19±6.54	88.33 ±4
11 d after diabetes onset						
Controls	69.69±3.57	66.34±4.42	63.49± 4	98.9 ±10.21	76.19 ±4.96	48.83 ± 3.74
Diabetic	58.88±3.32[a]	54.01±3.91	58.94±3.21	131.1±3.79[b]	65.01±4.23	51.02 ± 3.95

mRNA expression is shown as OD (arbitrary units). Values are expressed as mean ± SEM; n=7–8. The AVP mRNA quantification does not distinguish magnocellular from parvocellular cells. DG, Dentate gyrus. [a]*P < 0.05 vs. control.* [b]*P < 0.01 vs. control.*

and 11 d after diabetes onset. Moreover, we provided evidence that the up-regulation of adrenocortical ACTH receptors is an underlying mechanism responsible for the chronic hypercorticism in T1D. A better understanding of these mechanisms may open up new avenues for therapeutically useful strategies to normalize neuronal disturbances and improve cognitive disabilities of diabetic patients. Furthermore, the profound disturbance in the HPA axis regulation provides evidence for a role of B in diabetic neuropathology. Whether T1D leads to a more fragile state of the brain in which B excess may enhance the potential for damage and attenuate protective mechanisms, thus facilitating cognitive dysfunction and impair the ability to respond to stress, remains to be demonstrated.

In summary, in our mouse STZ model of T1D, the HPA axis readily reached a new setpoint characterized by high circulating B, low ACTH levels, and enhanced adrenocortical sensitivity. Surprisingly, ACTH levels were never elevated, also not at the onset of diabetes when hyperglycemia and later hypercorticism have developed. The up-regulation of ACTH receptors in the adrenal glands of STZ-induced diabetic mice might explain, at least in part, how hypercorticism is triggered and maintained. Moreover, the enhanced AVP mRNA in the PVN (also reported in the spontaneous T1D model, the NOD mouse) (60) and decreased MR mRNA in the *dentate gyrus* also may be considered manifestations of a profound disturbance in HPA axis regulation.

Acknowledgments

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CHAPTER 4

Neuronal and astroglial alterations in the hippocampus of a mouse model for type 1 diabetes

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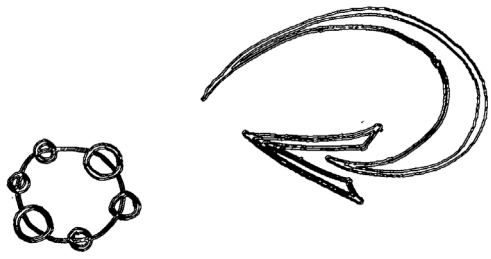

Abstract

The influence of diabetes mellitus on brain pathology is increasingly recognized. Previous contributions of our laboratory demonstrated in models of type 1 diabetes (nonobese diabetic and streptozotocin (STZ)-treated mice), a marked astrogliosis and neurogenesis deficit in hippocampus and increased expression of hypothalamic neuropeptides. In the present investigation, we further analyzed alterations of astroglia and neurons in the hippocampus of mice 1 month after STZ-induced diabetes. Results showed that these STZ-diabetic mice presented: (a) increased number of astrocytes positive for apolipoprotein-E (Apo-E), a marker of ongoing neuronal dysfunction; (b) abnormal expression of early gene products associated with neuronal activation, including a high number of Jun + neurons in CA1 and CA3 layers and *dentate gyrus*, and of Fos-expressing neurons in CA3 layer; (c) augmented activity of NADPH-diaphorase, linked to oxidative stress, in CA3 region. These data support the concept that uncontrolled diabetes leads to hippocampal pathology, which adjoin to changes in other brain structures such as hypothalamus and cerebral cortex.

DIABETES MELLITUS, regardless of its type, is associated with cerebral alterations in both human and animal models of the disease [5,23,44]. These alterations include abnormal expression of hypothalamic neuropeptides [11,57], hippocampal astrogliosis [59], decreased hippocampal synaptic plasticity [34,41], neurotoxicity, and changes in glutamate neurotransmission [8,20,67]. Diabetic patients are prone to moderate alterations in memory and learning [18,61] which occasionally may become severe [24] and to enhanced risk of stroke, depression, dementia, and Alzheimer's disease (AD) [3,17,23,28,43,44,48,49,65]. Recently, we have established that neurogenesis, the proliferation of new neurons in the adult brain, is strongly reduced in STZ-treated mice. Furthermore, this deficit has been successfully prevented by administration of estrogens to diabetic mice [60]. Hippocampal neuronal loss by apoptosis is present in animals suffering from long-term spontaneous diabetes (BB/W) in particular if complicated with ischemia [39,53]. However, such damage in the absence of appropriate treatment is not exclusive of hippocampus, since lesions and functional alterations of the cerebral cortex and hypothalamus have also been reported [2,11,48,57,62].

We previously found in two animal models of type 1 diabetes (T1D) an increased number of glial fibrillary acidic protein (GFAP)-reactive astrocytes in the hippocampal *stratum radiatum* [59]. These animal models include firstly the nonobese diabetic (NOD) mouse, which develops spontaneously the disease permitting analysis during the prediabetic and diabetic period. Secondly, the STZ-induced model, where changes are assessed after onset of the diabetes. In the present study, the STZ model is used for further study of markers for changes in astrocytes and neurons.

Astrocytosis is often related to neurodegenerative diseases and aging, in which neuronal dysfunction or damage can also be found [14,25,42,56]. Apo-E, an apolipoprotein primarily localized in astrocytes [19], plays a major role in various CNS disorders, particularly AD. Apo-E, involved in metabolism and lipid transport, is one of the amyloid-related proteins of AD cerebral amyloid plaques [30,54,66]. The Apo-Eε4 genotype is associated with a specific cognitive disadvantage in young women with T1D [16]. Moreover, individuals with both type 2 diabetes and the Apo-Eε4 allele show increased risk for AD [52]. Degenerating neurons can also express Apo-E after kainic acid treatment, while mild neuronal degeneration correlates with increased Apo-E levels in hippocampal astrocytes [26]. It should be mentioned, however, that Apo-E also is implicated in synaptic plasticity and learning [74]. Its accumulation in neurons following injury has been associated with neuronal survival [6].

Early gene products, such as the proteins encoded by proto-oncogenes of the c-fos and c-jun families, are the major components of the transcription factor activator protein 1 (AP-1), where they form different hetero- or homodimers. Suggestions have been presented that, in the CNS, these proteins may be involved in cell proliferation, gene transcription, stress response, regeneration, and cell death [46,55,63,75]. In the hypothalamus of diabetic rats, increased neuronal activity is observed in paraventricular nucleus (PVN), supraoptic nucleus (SON), and median preoptic nucleus (MnPO) measured by Fos specific staining [76]. On the one hand, there are evidences for an association

between immediate early gene products and apoptosis in different experimental models, including cerebral hypoxic-ischemic insult or injury [29,73]. On the other hand, c-Jun expression has been associated with neuronal survival [12,38,76]. Finally, some authors proposed c-fos and c-jun products as markers of a hippocampal subpopulation of neurons sensitive to excitotoxicity rather than as predictors of cell survival or death [55].

Another predictor of neuronal function is nitric oxide (NO), formed during the conversion of l-arginine in a NADPHd-dependent reaction by NO synthase (NOS) acting as an important intracellular signaling molecule [21]. NADPH-d is recognized as a histochemical marker for NOS [36,58]. Its neuronal isoform, nNOS, is found in neuronal cell bodies, dendrites and axons [7]. In the CNS, NO plays a major role in neuronal development and maturation [50]. Under aging or pathological conditions, such as neurodegenerative diseases, brain injury, or damage, there is increased NO production, with deleterious effects on neuronal function via enhanced oxidative stress.

Accordingly, the present study was designed to further asses the damage inflicted to neurons and astrocytes by experimental diabetes. For this purpose, three representative markers of neurodegeneration and neuronal dysfunction were measured in the hippocampus of mice with STZ-induced diabetes. These markers include the number of Apo-E immunopositive cells as a measure for astrogliosis and neurodegeneration, the level of immunoreactive immediate early gene products for the extent of neuronal activation and the activity of NADPH-diaphorase for the state of oxidative stress.

Materials and methods

Animals and treatment

12-week-old C57BL/6 female mice were housed under conditions of controlled humidity and temperature (22 ºC), with lights on from 07:00 to 19:00 h at the facility of the Institute of Biology and Experimental Medicine, Buenos Aires. Experimental procedures followed the NHI Guide for the Care and Use of Laboratory Animals (Assurance Certificate #A5072-01). Mice received a single i.p. dose of 200 mg/kg STZ (Sigma, St. Louis, MO, USA) in 0.5 M sodium citrate buffer or vehicle. Two days after injection, glycosuria was determined using Keto-Diastix (Bayer Diagnostics, Argentina). Following a positive urine test, mice were bled by retro-orbital puncture and blood glucose levels were evaluated using Accutrend (Roche Diagnostics Mannheim, Germany), and quantitatively measured using colorimeter (Accutrend GC, Boehringer Mannheim, Germany). Animals with glycemia higher than 11 mM were classified as overtly diabetic. One month after STZ or vehicle injection, 16-week-old animals were used for immunocytochemistry and NADPH diaphorase (NADPH-d) histochemistry.

Mice were anesthetized with Ketamine (60 mg/kg i.p.) and perfused intracardially with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.2. Brains were removed, fixed in the same fixative during 4–6 h, incubated overnight

in 15% sucrose in PB at 4 ºC, frozen on dry ice, and stored at -80 ºC until use. Coronal sections (s) (16–30 μm) containing hippocampus [51] were made in a cryostat, mounted on gelatin-coated slides and refixed by immersion in 2% w/v paraformaldehyde in PB during 6 min at 4 ºC and washed twice with PB for 10 min.

Apolipoprotein-E immunocytochemistry

The s (30 μm) were washed in 0.05M Tris-buffered saline (TBS), preincubated in 10% (v/v) goat serum for 1 h at room temperature (RT) and incubated overnight at 4 ºC with the primary Apo-E polyclonal antibody made in rabbit (kindly provided by Dr. NJ Pearce, SmithKline Beecham, UK, see [26]) in 1/5000 dilution in TBS 0.2%, Triton-X 100, 2% goat serum. After several washes with TBS, the s were incubated in goat anti-rabbit serum (1/200) in TBS 0.2% Triton X-100 for 2 h and processed following the ABC kit (Vector Labs., CA, U.S.A.) instructions. 3,3'-diaminobenzidine (DAB) was used for development at 0.5 mg/ml, 0.05% H_2O_2 at RT. After dehydration with graded ethanols and xylene, the slices were mounted with Permount. Nonspecific staining was assessed in the absence of primary antibody; the specific labeling was characterized as mentioned elsewhere [26]. The number of cells expressing Apo-E per area ($65x10^3$ μm^2) was determined in the *stratum radiatum* below the CA1 subfield of the dorsal hippocampus using computerized image analysis. Labeled cells were investigated in the mentioned area of both hippocampal sides from five to six slices per animal, using five animals per group. Positive cells were identified as reactive astrocytes, based on co-localization between glial fibrillar acid protein (GFAP) and Apo-E by immunocytochemistry (not shown).

Early genes products (Fos and Jun) immunocytochemistry

Briefly, the protocol used was as follows: brain s were incubated with Fos H-125 polyclonal rabbit antibody (Santa Cruz, Biotechnology, San Diego, CA, USA) 1/3000 in phosphate buffer saline (PBS) 0.15% Triton X-100 overnight at RT or Jun H-79 polyclonal rabbit antibody (Santa Cruz Biotechnology, USA) 1/500 in the same buffer, overnight at 4 ºC. After two washes with PBS, slices were incubated with goat anti-rabbit serum (1/200) in PBS 0.15% Triton X-100 during 1 h at RT, and processed following the ABC kit instructions (Vector Laboratories, Elite, ABC reagent). Development was carried out using 1 mg/ml DAB 0.01% H_2O_2 during two min at RT. Finally, s were dehydrated in graded ethanols and xylene and mounted with Permount. To assess nonspecific staining due to the immunoprocedure, some s were incubated replacing the primary antibody by no immune rabbit serum. Cells exhibiting Fos and Jun immunolabeling were identified as neurons based on their immunocytochemical reactivity towards the marker somatostatin. Although somatostatin is not an universally accepted marker for neurons, it has been used to stain hippocampal neurons [1]. In this case, somatostatin-immunoreactive cell area (65.9 ± 4.1 μm^2, $n = 21$), was identical to cell area of Fos/Jun-positive cells counterstained

with cresyl violet (63.3±2 μm^2, n = 19).

NADPH-diaphorase histochemistry

A slight modification of the method of Vincent and Kimura [71] was employed to determine NADPH-d activity as previously described [36,58]. The latter was used as a marker for NOS, because while neurons can express several NOS isoforms during pathological conditions, all isoforms share NADPH diaphorase activity [69,70]. Cryostat s (16 μm) were incubated in a solution of 0.1M Tris HCl buffer pH 7.4 containing 0.3% (v/v) Triton X-100, 0.2 mg/ml of nitroblue tetrazolium, 2.7 mg/ml l-malic acid and 1 mg/ml β-NADPH. After 60–90 min at 37 °C in the darkness, the reaction was stopped by two washes in PBS at RT. s were then dehydrated briefly in ethanol, dried and coverslipped with Permount. To assess non-specific staining due to the procedure, some s were incubated in buffer without β-NADPH. NADPH-diaphorase active cells were identified as neurons based on immunoreaction with an anti-somatostatin antibody, as shown above for Fos and Jun-expressing cells.

Measurement of hippocampal volume

Cresyl-violet stained serial slices (50 μm, every 8 slices), obtained with a vibratome, were used to estimate the volume using a computer-assisted image analysis (Optimas Bioscan 4.2) on the basis of the Cavalieri principle. Structures were outlined, and the computed areas were summed and multiplied with the inter distance and with the thickness of the slice. Volumes are expressed as mm^3 per 750 μm.

Computerized image analysis

A computerized image analysis (program Bioscan Optimas II; Edmonton, WA, USA) equipped with a VT-C330N video camera was used for quantitative analysis [59]. Digitized images of tissue s containing hippocampus were displayed on the video screen under identical lighting conditions. Using this program, we set up a threshold for positive cell area, and within these area limits, nucleated cells exhibiting Apo-E labeling were selected for our study. For early genes products, we quantified the number of immunoreactive nuclei in CA1, CA3 and *dentate gyrus* (DG) areas. For NADPH-d labeling, we quantified the intensity (as inverse logarithm of gray intensity per area, ILIGV/area: LIGV) of the reaction in CA1 and CA3 subfields [36].

Statistics

Statistical analysis was performed using Student's t test with two-tailed P value. Differences are considered significant when the two-tailed P value is < 0.05.

Results

Pathophysiology

The animals were used 4 weeks after STZ injection. At this time period, blood glucose was high and comparable to animals bled 48 h after diabetes induction. Diabetic mice showed marked hyperglycemia (15.8±2.0 mM), polydipsia (control water intake: 3±0.5 ml/day; diabetics 5.2 ml/day, $P < 0.05$), and increased food intake (controls: 0.6±0.02 g/day, diabetics: 0.72±0.03 g/day, $P < 0.05$). Initial body weight (experimental day 1) was similar in control (26.5±1.1 g) and diabetic mice (26.1±0.5 g) but controls were significantly heavier than diabetic animals at the conclusion of the experiment (30.0±1 g vs. 20.3±1.4 g, $P < 0.01$). Therefore, at the time of killing, STZ-treated mice showed characteristic signs of overt diabetes.

Hippocampal volume was not altered in diabetic mice

Measurement of hippocampal volume showed no significant difference between control and STZ-induced diabetic mice: controls: 2.203±0.076, diabetic: 2.317±0.051 mm^3 (n=5 animals in each group).

More Apo-E+ cells in the hippocampus of STZ-treated diabetic mice

The immunocytochemical features of immunoreactive Apo-E cells present in the *stratum radiatum* of 16-week-old vehicle- or STZ-treated mice, under the CA1 region, are given in Figs. 1A and B, respectively. In both groups of mice, Apo-E immunostaining was localized specifically in cells with a spider-like shape typical of astrocytes, exhibiting a GFAP-positive phenotype. Moreover, Apo-E+ cells appear more numerous in the STZ- than in the vehicle-treated group. We therefore quantified the number of Apo-E+ cells in both groups of mice by computerized image analysis (Fig. 2). One month after STZ treatment, the number of Apo-E+ astrocytes was significantly increased in the STZ-treated group as compared to the vehicle-treated group ($P < 0.001$, $n = 5$).

The number of astrocytes immunoreactive for Apo-E was in accordance with data obtained using a GFAP antibody. In this case, a 1.4-fold increase in cell number was obtained for both Apo-E-positive and GFAP-positive astrocytes in diabetic compared to control mice.

Enhanced expression of early gene products (Jun and Fos) in the hippocampus of STZ-treated diabetic mice

Cellular activation was studied by immunocytochemistry using specific antibodies against Jun and Fos. Immunoreactivity for early gene products was localized in neurons but absent from astroglial cells in white matter regions of hippocampus. Fig. 3 shows the

photomicrographs corresponding to Jun in the hippocampal CA1, CA3, and DG neurons and to Fos in CA3, respectively. Numerous positive nuclei were observed in each case in the STZ-treated group compared to the vehicle-treated group. Quantitative assessment of these data by computerized image analysis is shown in Fig. 4. When STZ-treated mice were compared to vehicle-treated mice, the numbers of positive nuclei were significantly higher in diabetic mice, as follows: for Jun (Fig. 4, top), in neurons corresponding to the different hippocampal regions studied (CA1, CA3, and DG) ($P < 0.01$ in each case, $n = 6$) and for Fos (Fig. 4 bottom), in CA3 neurons only ($P < 0.05$, $n = 4$) but a tendency toward increased number of Fos+ nuclei was also observed in CA1 and DG subfields in the diabetic group. Preliminary data in our short-term model of diabetes showed that the total number of hippocampal neurons in CA1 and CA3 regions presenting somatostatin immunoreaction was similar in control and diabetic mice. Since the presence of both Jun and Fos were linked to development of neurodegeneration [73], we then studied NAPDH-d as a marker of oxidative stress.

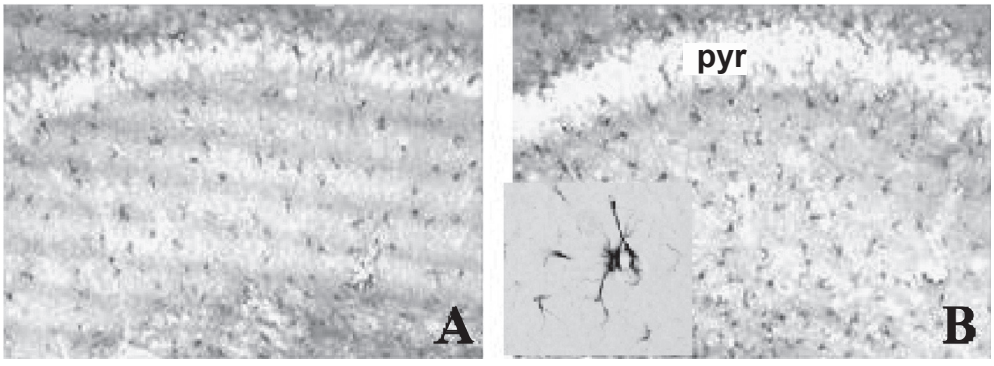

Fig. 1. *Photomicrographs showing Apolipoprotein E (Apo-E)-positive cells in hippocampal stratum radiatum from 16-week-old female control C57BL/6 (A), STZ-diabetic mice (B). Note the numerous positive cells in B, pyr: pyramidal cell layer, magnification: 400X. In B, inset showing Apo-E positive cell at higher magnification.*

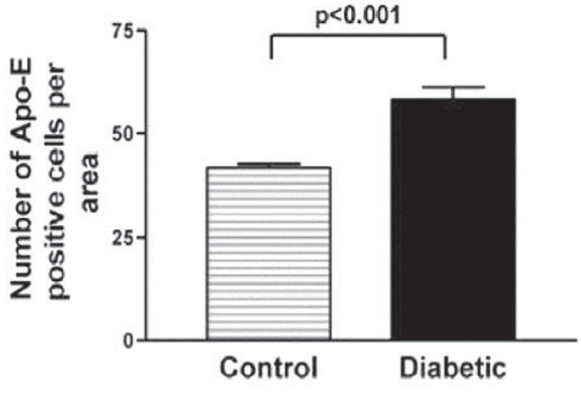

Fig. 2. *Quantitation by computerized image analysis using Optimas Bioscan Software of the number of Apo-E positive cells per area (65 x 10^3 μm^2) in hippocampal stratum radiatum region from control C57BL/6 and STZ diabetic mice.*

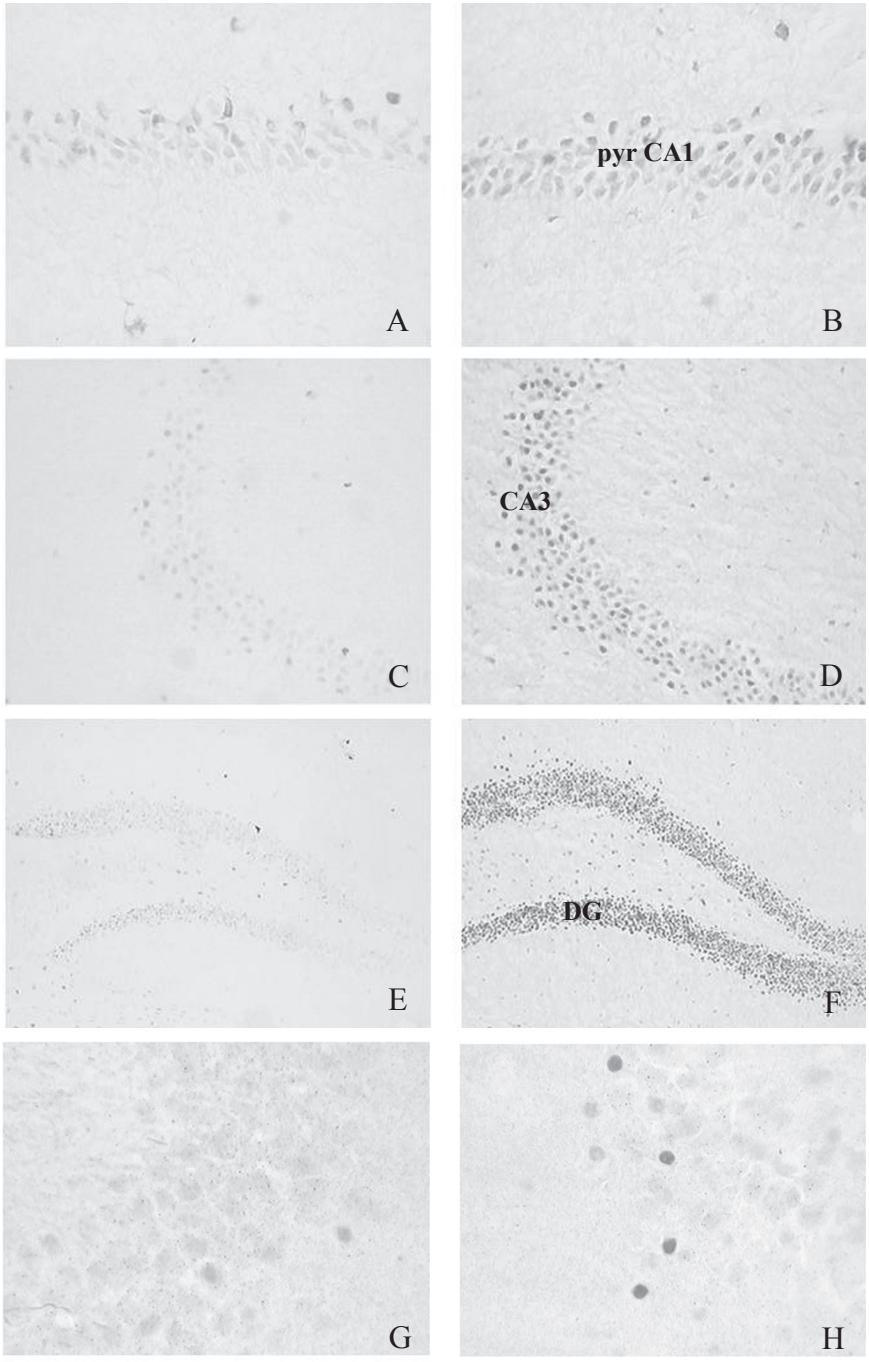

***Fig. 3**. Photomicrographs showing Jun (A–F) and Fos (G–H) immunoreactive cell nuclei of hippocampal neurons from control (A, C, E, G) and STZ-diabetic mice (B, D, F, H). CA1 area is shown in A, B, CA3 in C, D and G, H, Dentate Gyrus (DG) in E, F photomicrographs. Magnification: A–B: 200X; C–F: 100X and G–H: 400X; pyr CA1: pyramidal cell layer corresponding to CA1 hippocampal region, DG: dentate gyrus.*

Increased NADPH-diaphorase activity in the hippocampus of STZ-treated diabetic mice

Figs. 5A and B shows an example of NADPH-d histochemistry in the hippocampal CA3 region of both groups of mice. Once again, a marked difference could be observed between both groups, as shown by computerized image analysis of staining intensity (Fig. 6). There was a marked increase of enzyme activity in neurons of both the CA1 and CA3 regions, but this difference reached significance only in CA3 ($P < 0.005$, $n = 6$).

Discussion

In the present study, some molecular parameters were investigated in the hippocampus of STZ-treated diabetic mice that could reflect functional abnormalities of astrocytes and neurons. Previously, we have reported a substantial reduction of hippocampal neurogenesis in STZ-diabetic mice [60] and increased number of GFAP-immunoreactive astrocytes in the spontaneous model of T1D, the NOD mouse, and the pharmacologically-induced STZ mouse model [59]. Here, we showed, at the astrocyte level, increased Apo-E immunoreactivity in the CA1 region. At the neuronal level, we observed an enhanced expression of the immediate-early gene product Jun in the CA1, CA3, and DG regions of the hippocampus. In addition, a high amount of Fos-positive nuclei and high NADPH-d activity was found in the CA3 region. These findings, as well as previous, indicate that 1-month exposure to uncontrolled diabetes can alter the hippocampus. On the one hand, the alteration might reflect the regenerative capacity of the brain: Apo-E, Fos and Jun, and the NO may contribute to the recovery of neuronal processes. On the other hand, the Apo-E, Fos, Jun and NO increases may represent responses to a mild neurodegeneration. Below we will discuss the arguments involving these possibilities.

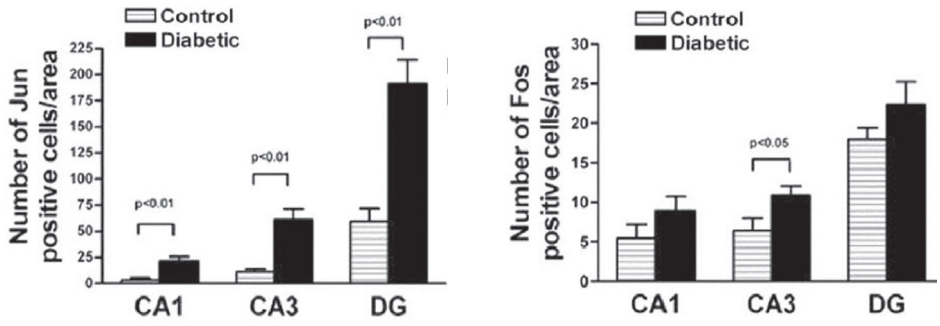

Fig. 4. *Quantitation using computerized image analysis of early genes products in hippocampus of control and STZ-diabetic mice. Number of Jun immunoreactive cell nuclei in CA1, CA3 and DG hippocampal regions from vehicle-treated and diabetic STZ-treated mice (top). Number of Fos immunoreactive cell nuclei in CA1, CA3, and DG hippocampal regions from vehicle-treated and diabetic STZ-treated mice (bottom).*

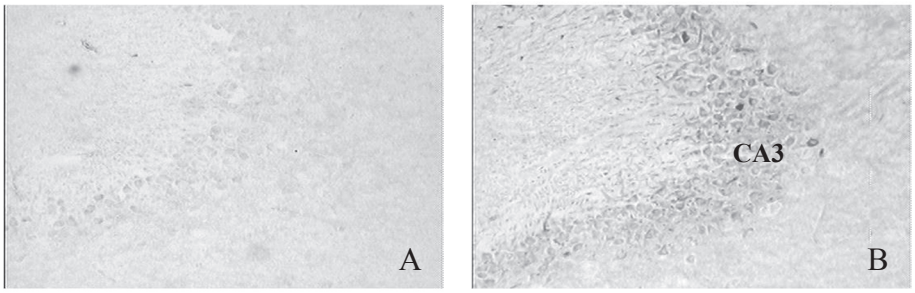

Fig. 5. *NADPH diaphorase histochemical staining in CA3 hippocampal region from control (A) and diabetic-STZ treated mice (B). Magnification: 200X.*

Apo-E is synthesized by astrocytes, oligodendrocytes and ependymal layer cells [6]. It has been suggested that Apo-E has a neurotrophic role that may be needed for synaptic plasticity and learning [74], ApoE also seems involved in the removal of cell debris, because degeneration products were shown to remain in the hippocampus of Apo E deficient-mice [15,68]. Under normal conditions, Apo-E is thought to enter neurons (rather than to be synthesized by them) and this uptake in neurons is enhanced during repair after injury. Peripheral and CNS injury is associated with a strong increase in Apo-E expression in non-neuronal cells [6]. However, after kainic acid-induced hippocampal neurodegeneration, the intensity and cellular distribution of Apo-E is dependent on the severity of neuronal injury, which correlate with the dose of toxin administered. Indeed, mice that developed mild neuronal degeneration restricted to a subset of hippocampal neurons show increased Apo-E expression in the hippocampus concomitant with GFAP immunoreactivity and mild microgliosis. In contrast, mice with severe hippocampal neuronal injury show intense Apo-E expression in degenerating neurons and increased Apo-E mRNA levels in clusters of CA1 and CA3 pyramidal neurons [6,26].

Since no signs of pyramidal cell death are present in Apo-E+ neurons of kainic acid-treated mice, it has been suggested that neuronal Apo-E expression may be part of a rescue program to counteract neurodegeneration [6,32]. In humans, Apo-E immunoreactivity has been described in cortical and hippocampal neurons of AD and aged control subjects [27,45]. In the context of this work, it is worth noting the strong association between type 2 diabetes and AD, as well as between type 1 diabetes and changes in cognition among carriers of the Apo-Eε4 allele [16,37,52]. The joint effect of diabetes and Apo-Eε4 is synergistic, causing more than a 5-fold increase in the risk for AD. Therefore, taking into account all these data, it seems likely that an exposure to T1D for 1 month in the STZ-mouse model can induce moderate hippocampal disturbances, since Apo-E expression is increased only at the astrocyte level. The effects on Apo-E expression might reflect a more general effect upon astrocytes, since the total number of these cells, according to GFAP immunocytochemistry, was increased in the *stratum radiatum* of STZ-diabetic mice [59]. These alterations seem to be early events following diabetes induction, considering that a 30% increase in astrocyte number in the *stratum radiatum* is measured 48 h after STZ treatment (unpublished data), which increased to 50% of control levels after 4 weeks of uncontrolled diabetes. This is also the case in NOD mice with a genetic

type of T1D [59]. Regarding the significance of increased Apo-E expression, further studies are needed to elucidate if this change reflects an incipient neurodegeneration or represent a response underlying recovery.

In addition to increased astrocyte reactivity [59] and increased Apo-E immunoreactivity in astrocytes (this paper), the induction of T1D also resulted in changes in Jun- and Fos-positive nuclei in neuronal populations of the CA1, CA3 and DG hippocampal areas. Immediate early genes (IEG) products have been extensively investigated in relation to neuronal cell death. In in vitro systems, using NGF-deprived sympathetic neurons IEGs are indicative of neuronal cell death taking place during postnatal development. Cell death linked to IEG production also occurs under in vivo situations after brain injuries, such as hypoxic ischemia and kainic acid administration [29,31,55,73]. Accordingly, several of these studies show increased expression of Fos and Jun *in situ*ations associated with neuronal cell death, aging and impaired cognition [64]. Moreover, the presence of Fos and Jun-like immunoreactivity has been reported in neurons of the AD brain [47]. While these studies imply a functional role of early gene products in neuronal cell death, other studies questioned this interpretation, since many cells that expressed Fos and Jun during progression of excitotoxic lesions due to hypoxic-ischemia or kainic acid administration survive the injury, particularly at the hippocampal level [29,55,73]. It has therefore been suggested that: (1) the biological effect of the Jun N-terminal kinase (JNK)/c-Jun signalling pathway depend on the neuronal type and stage of maturation [13]; (2) Fos and Jun neuronal response might not contribute to cell death but rather to cell repair and/or regenerating processes [29]. Taken together, increased Fos- and Jun-positive cells, particularly in the absence of neuronal Apo-E expression, suggested that neuronal derangement was emerging in the hippocampus of 1-month diabetic animals.

A second neuronal abnormality found in T1D was NADPH-d (or nNOS) which showed a significant increase in the CA3 hippocampal field of STZ-treated diabetic mice. Normally, NADPH-d is present in the intact brain only in selected populations of neurons but absent from glial cells. It is only after a severe brain lesion that astrocytes display intense NADPH-d activity [72]. With regard to diabetes, 4 weeks after STZ injection into rats, increased expression of nNOS mRNAs and NADPH-d staining are found in the hypothalamus, which are partly suppressed by insulin treatment [62]. As discussed by others, the significance of the up regulation of NO during brain lesions is ambiguous. NO could have a protective effect on damaged neurons, being a major factor of synaptogenesis and axonal sprouting and might be associated with neuronal regeneration. But it may also be involved in neuronal cell death [35]. These authors recently showed using nNOS-KO mice that local release of NO following peripheral nerve injury is a crucial factor in degeneration and/or regeneration.

With regard to the STZ effects on the brain, and particularly, on the hippocampus, it has already been stressed that the type and the extent of the alterations are a function of both the severity of hyperglycemia as well as its duration [5]. First, alterations of Apo-E-positive astrocytes, as those described here, may accompany a mild neuronal activation that could reflect a stage of reactivity or adaptation to metabolic disturbances. Concomitantly, the diabetic process can affect neuronal renewal [33]. If the deleterious stimulus continues, then signs of neurodegeneration, alterations of neuronal morphological parameters, and neuronal cell death would occur. In this sense, a time-related apoptosis

was observed in a genetic model of T1D, the BB/W rat [39]. These authors showed beside an increased number of TUNEL positive cells, stimulated levels of caspase-3 activity, internucleosomal DNA cleavage and induction of proapoptotic genes in hippocampus of 8-month but not in 2-month diabetic rats.

In addition to hyperglycemia, other disturbances may contribute to the changes observed in the hippocampus. In this respect, it is known that diabetic animals show aberrant activity of the glucocorticoid system, including increased circulating corticosterone levels, lack of periodicity, hypersensitivity to stress, and down-regulation of hippocampal glucocorticoid receptors [10,22,41]. In STZdiabetic mice and in NOD mice, we have shown increased levels of vasopressin mRNA in the hypothalamic paraventricular nucleus, indicative of persistent stress [57]. Thus, the highly stressful condition of diabetic animals might enhance the vulnerability of brain areas with a high degree of plasticity such as the hippocampus [9]. Therefore, it is likely that anatomical and phenotypical features observed in the hippocampus underlie the impairment of cognitive performance of diabetic animals [3,17,23]. Along this line, preliminary studies demonstrated that the exploratory activity in the closed arm (non-conflicting environment) of the elevated asymmetric plus-maze set up is decreased in STZ-diabetic mice (Saravia *et al*,unpublished).

The present study completes the view of the hippocampal disturbances that we previously described in T1D mouse model [59,60]. The observed changes are not fully conclusive of hippocampal neurodegeneration. Only after prolonged untreated diabetes severe hippocampal neurodegeneration with signs of apoptosis may develop [39] or appear as secondary complications of the disease [53]. Moreover, the hippocampal disturbances described here and elsewhere after 1 month of diabetes could not *a priori* be attributed to hippocampal volume changes because no significant differences on volume measurement were found between control and diabetic mice. The fine hippocampal disturbances showed in this model of T1D could be among the primary basic mechanisms underlying the well-known brain alterations associated with diabetes. Such alterations, like the dysregulation of the hypothalamo–pituitary–adrenal axis [10,40,41], increased risk of stroke and dementia [5] and cognitive deficits are also found during ageing. Learning and memory, for example, two processes in which hippocampal synaptic plasticity is involved, can be disturbed in both diabetic humans and animals [4,8,20,34,67]. Moreover, in humans with long-standing diabetes, marked deficits in cognition were also reported [24]. Therefore, data obtained in the mouse hippocampus may be useful to interpret the functional deficits of diabetic patients.

Acknowledgments

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Chapter 5

Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice.

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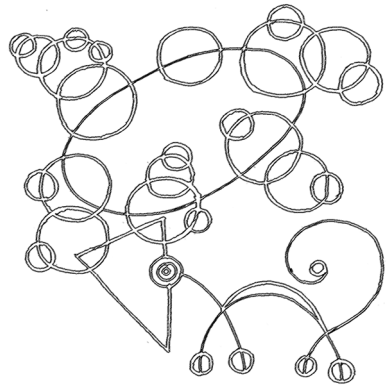

Abstract

Type 1 diabetes is a common metabolic disorder accompanied by an increased secretion of glucocorticoids and cognitive deficits. Chronic excess of glucocorticoids *per se* can evoke similar neuropathological signals linked to its major target in the brain, the hippocampus. This deleterious action exerted by excess adrenal stress hormone is mediated by glucocorticoid receptors (GR). The aim of the present study was to assess whether excessive stimulation of GR is causal to compromised neuronal viability and cognitive performance associated with the hippocampal function of the diabetic mice. For this purpose, mice had Type 1 diabetes induced by streptozotocin (STZ) administration (170 mg/kg, ip). After 11 days these STZ-diabetic mice showed increased glucocorticoid secretion and hippocampal alterations characterized by: 1) increased GFAP positive astrocytes as a marker reacting to neurodegeneration, 2) increased c-Jun expression marking neuronal activation 3) reduced Ki-67 immunostaining indicating decreased cell proliferation. At the same time, mild cognitive deficits became obvious in the novel object-placement recognition task. After 6 days of diabetes the GR antagonist mifepristone (RU486) was administered twice daily for 4 days (200 mg/kg, p.o.). Blockade of GR during early Type 1 diabetes attenuated the morphological signs of hippocampal aberrations and rescued the diabetic mice from the cognitive deficits. We conclude that hippocampal disruption and cognitive impairment at the early stage of diabetes are caused by excessive GR activation due to hypercorticism. These signs of neurodegeneration can be prevented and/or reversed by GR blockade with mifepristone.

TYPE 1 DIABETES (T1D) is an autoimmune disease that results in the permanent destruction of insulin-producing beta-cells of the pancreas. To study the disease, a commonly used rodent model is generated by injecting streptozotocin (STZ). This toxin destroys the insulin-producing cells producing diabetes (Rees and Alcolado, 2005). The impact of T1D on the central nervous system is well documented. T1D patients show impairments in explicit memory, problem solving, and intellectual development (Ryan and Williams, 1993; Ryan, *et al* 1993; Kramer, 1998; Parisi and Uccioli, 2001; McCarthy, *et al* 2002; Schoenle, *et al* 2002). In the STZ-diabetic rodent model, the severity of the cognitive deficits is related to the duration of diabetes (Kaleeswari, *et al* 1986; Bellush and Rowland, 1989; Flood, *et al* 1990; Mayer, *et al* 1990; Biessels, *et al* 1996, 1998; Popovic, *et al* 2001). Some studies have revealed structural and functional abnormalities, particularly in the hippocampus, such as impaired long-term potentiation, synaptic alterations (dendritic spine densities and LTP), degeneration and neuronal loss (hamsters: Luse, 1970; rats: Bestetti and Rossi, 1980; Biessels, *et al* 1996; Gispen and Biessels, 2000; Magarinos and McEwen, 2000; McEwen, *et al* 2002, mice: Saravia, *et al* 2002). In the hippocampus of STZ-mice we have previously demonstrated: 1) astrogliosis, as evidenced by increased number of GFAP positive cells, suggesting neuronal suffering, 2) increased immediate early gene expression such as elevated c-Jun immunoreactive neurons, a sign of neuronal activation, and 3) decreased neurogenesis in the *dentate gyrus* (DG), indicating that newborn cells are vulnerable to damage in diabetes (Saravia, *et al* 2002; Revsin, *et al* 2005). Overall, these alterations suggest mild hippocampal neurodegeneration in the STZ-treated mice.

In rodents the effects of the glucocorticoid corticosterone on learning and memory processes are mediated by mineralocorticoid and glucocorticoid receptors (MR and GR), which are highly expressed in limbic brain areas. Glucocorticoids secreted after stress promote consolidation of a stressful event via transient activation of GR (Oitzl and de Kloet, 1992, Joëls, 2006). In contrast, sustained activation of GR by chronically elevated glucocorticoids impairs hippocampal function and memory processes (McEwen and Sapolsky, 1995). However, cognitive performance is improved during chronic blockade of GR with the glucocorticoid antagonist mifepristone (RU486) (Oitzl, *et al* 1998; Conrad, 2006). MR is neuroprotective and stimulates hippocampal function (Joëls, *et al* 2008). It mediates the role of corticosterone in the appraisal of novel situations, behavioral reactivity and affective responses (Oitzl and de Kloet, 1992; Oitzl, *et al* 1994; Rozeboom, *et al* 2007), and enhances the performance in spatial hippocampal-dependent cognitive tasks (Ferguson and Sapolsky, 2007, Lai, *et al* 2007). Thus, a balanced activation of MR and GR is crucial for optimal cognitive performance.

In diabetic animals acute cerebral dysfunction resulting from hyper- and hypoglycemic episodes was described (Biessels, *et al* 1994; Cryer 1994). In addition, chronic hypercorticism triggered and maintained by enhanced adrenocortical sensitivity in STZ-mice, and morphological alterations of predominantly the hippocampus were reported (Fitzpatrick, *et al* 1992; Durant, *et al* 1993; Scribner, *et al* 1993; Saravia, *et al* 2002 and 2004; Revsin, *et al* 2005 and 2008). A fundamental question in the neuropathology

of T1D is, therefore, whether aberrant glucose metabolism and insulin deficiency and/or glucocorticoid excess and excessive GR activation may cause the functional and morphological signs of neurodegeneration and cognitive impairment. Previous reports described the central roles of glucose and insulin in the STZ-induced animal models of T1D (Sima and Li, 2005; Inouye, *et al* 2005; Chan, *et al* 2005; McNay, *et al* 2006).

Recently, chronic hypercorticism was found to be responsible for the functional and morphological degeneration in hippocampus of rats and mice suffering from full-blown T1D (Stranahan, *et al* 2008). The study showed that impaired LTP, deficits in cognitive performance and reduced neurogenesis were normalized in diabetic rodents after adrenalectomy and corticosterone replacement at physiological concentrations (Stranahan, *et al* 2008). The objective of the present study is to evaluate whether these neurodegenerative effects of glucocorticoids in T1D mice were due to excessive activation of GR. We hypothesize that the STZ-induced hypercorticism of diabetic mice and concomitant continuous activation of GR may change molecular markers of hippocampal morphology and cognitive perfromance. We have used the GR antagonist mifepristone (RU486) in order to clarify the role of continuous GR activation in the hippocampus of STZ-diabetic mice. Hippocampal integrity was based on markers for cellular proliferation and activation. For hippocampal function spatial memory tasks were used, specifically the novel object-placement recognition task. The data demonstrate protection of hippocampal integrity and improved cognitive performance by a brief GR antagonist treatment during the onset of diabetes in the STZ-diabetic mice.

Materials and Methods

Animals

12 weeks-old C57Bl/6 male mice (Janvier, NL) were grouped housed 3 to 4 per cage in humidity (55±5 %) and temperature (23±2 ºC) controlled conditions, with 12-12 light-dark hours cycle (lights on at 8am) at the animal facility of the LACDR, Leiden. Food and water was provided *ad libitum*. Animal experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University.

Experimental schedule

Mice received a single i.p. dose of 170mg/kg of streptozotocin (Sigma, St. Louis, MO, USA) in 0.5M sodium citrate buffer or vehicle; 48hrs after injection glucose levels in urine (Diabur-Test 5000, Roche, Germany) indicated diabetes. Mice with blood glucose levels (Accu-Chek Compact, Roche, Germany) higher than 11mM were classified as overtly diabetic. Six experimental groups (n=6-8 mice per group) were designed as follows: 1) control and diabetic groups with 6 days of diabetes; 2) control and diabetic group with 11

days of diabetes vehicle-treated; 3) control and diabetic group with 11 days of diabetes mifepristone-treated. Six days after diabetes onset at 9am, mice received every 12hrs 100µl of mifepristone (11β-(4-Dimethyl-amino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one, Corcept Therapeutics, CA, USA) for 4 consecutive days (200mg/kg body weight, per os) dissolved in 0.25% carboxymethylcellulose and 0.2% Tween20 in 1ml NaCl 0.9%, or vehicle. This dose of mifepristone ensures a complete blockade of the GR for at least 12hrs (Dalm, *et al* 2008). Mifepristone displays antiglucocorticoid and antiprogestagen activity. However, in conditions of chronic elevated glucocorticoid levels, such as occurring in T1D mice, the antiglucocorticoid is most prominent. Moreover, mifepristone has been used as antiglucocorticoid in animals models for stress (Krugers, *et al* 2007; Mayer, *et al* 2006; Oomen, *et al* 2007), drugs of abuse (Dong, *et al* 2006) and electroconvulsive therapy (Nagaraja, *et al* 2007). At day 11 of diabetes at 8am, the behavioral experiment was started at a time GR blockade during the task is still ensured. Mice were sacrificed between 10am and 12am for all the experiments (see Figure 1a).

Novel object-placement recognition (NOPR) task

Based on locomotor activity and the time exploring the objects during the last habituation trial (each object was explored approximately equally long), mice were divided into two groups that received either STZ or vehicle injection.

Apparatus and objects

Object exploration was assessed in an open field (50x50x40 cm; gray polyvinylchloride). Mirrors were placed on the walls of the open field to improve the visual analysis of the exploratory behavior of the mice. The illumination density was approximately 50 lux at the center of the open field.

We used two identical plastic bottles filled with water as objects (0.5 liter without labels). After each trial, the apparatus and objects were thoroughly cleaned with a 75% ethanol solution to remove or spread odor cues.

Procedure

A scheme of the experimental design is presented in Figure 1. Figure 1b shows the handling and habituation procedure adapted from Dere *et al* 2005.

At the start of each trial, mice were picked up from the base of the tail and placed into an opaque cylinder (10cm diameter; 20cm high) in the center of the open field. After 10 seconds, the cylinder was lifted and the mice were allowed to explore the environment. Depending on the experimental condition, the two bottles were present or absent. At the end of each trial, the mice were removed from the open field by a grid and returned to the home cage. This procedure prevents the mice becoming stressed by the experimenter.

On day 11 of diabetes the NOPR test was performed (Figure 1c). In the sample trial the two objects were present in the open field: one in the north-west zone (NW) and one in the south-west zone (SW). After 10min exploration, the mouse was returned to its home cage for 50min. For the consecutive 10min test trial (T2), the SW object was re-located to the east zone (E).

Computerized image analysis

Exploration of an object is defined when the mouse is in the vicinity of an object at less than 2cm and in physical contact with it. Contact was only counted as exploration when the mouse touched the object with the snout, forepaws or vibrissae (Observer 4.1, Noldus). The parameters of exploration are based on Dere *et al* 2005.

Analysis of general locomotor activity and behavior was performed by EthoVision3.0 (Noldus Information Technology, Wageningen, The Netherlands) using steps of 5 samples *per se*cond. The open field was divided into 9 zones (Figure 1), and total distance moved (cm), mean velocity (cm/s), latency of first entry in each zone (seconds) and time per zone (percentage) was measured.

Calculations

The exploration of the two objects was calculated for the sample and test trials. The percentage of preference for exploring the re-located object was calculated as follows:

$$\frac{\text{exploration time of re-located object (T2)}}{\text{sum of exploration of both objects}} \times 100$$

Immunocytochemistry for GFAP and Ki-67

Under pentobarbital sodium salt anesthesia (Nembutal 150µl i.p., CEVA Sante Animale BV, The Netherlands) mice were intra-cardially perfused (20ml of 0.9% NaCl followed by 40ml of 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS), pH7.4), the brains were removed, kept for 2hrs in the same fixative and incubated overnight in 30% sucrose in PBS at 4^{o}C. Thereafter, the brains were frozen on dry ice and stored at -80^{o}C until processing.

Astrocytes and proliferative cell markers (GFAP and Ki-67) were measured in coronal brain cryosections (20µm) from the dorsal hippocampus (bregma -1.7 to -2.06mm; Paxinos and Frankling, 2001). Four to six sections from each brain were mounted per glass (SuperFrost Plus glasses, Menzel-Glaser, Germany) and stored at −80°C. After washes with 0.1M Tris buffered saline pH7.6 (TBS) the sections were placed in plastic jars filled with citrate buffer (0.1M for GFAP and 0.01M for Ki-67, pH6), microwaved for 5min at 800W, 400W and 250W, and cooled down to room temperature (RT) for 30min.

Ki-67 immunostaining follows the previously published by Heine *et al* (2004). For GFAP nonspecific binding was prevented by 10% normal goat serum (NGS)/0.3% Triton X-100 in 1M TBS for 30min at RT. Thereafter, sections were incubated with the primary antibody rabbit polyclonal anti-GFAP (1:600, Dakoctyomation, The Netherlands) diluted in 2% NGS/0.2% Triton X-100 in 1M TBS (TBS+) for 1hr at RT and overnight at 4°C. For negative control, the first antibody was omitted. The next day after rinses, sections were incubated with biotinylated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, The Netherlands) in TBS+ for 1hr at 37°C. After 45min incubation with ABC kit (1:800, Vector Labs., CA, USA), 3,3'-diaminobenzidine (DAB) 0.5 mg/ml, 0.05% H_2O_2 at RT was used for development. After dehydration with graded ethanols and xylene, the sections were mounted with Entellant.

Sections stained for Ki-67 were additionally counterstained with 0.5% cresyl violet, with unbiased stereological sampling and quantification in every 10th hippocampal section according to Saravia *et al* (2004) and Oomen *et al* (2007). The number of cells expressing GFAP per area ($31x10^4$ mm^2) was determined in the *stratum radiatum* below the CA1 using computerized image analysis (Olympus Soft Imaging). Labeled cells were bilaterally counted in four to six sections per animal, using 4 to 8 animals per group as indicated.

Western Blot for c-Jun

Western blot was performed as decribed by Vreugdenhil *et al* (2007) in decapitated separated groups. The same amount of protein (25µg cell lysate) from mouse hippocampal tissue (6 mice per group) was used. Polyclonal anti-c-Jun primary antibody (1:500, Santa Cruz Biotechnology, CA, USA) and monoclonal anti-α-tubulin DM1A (1:1000; Sigma–Aldrich, The Netherlands), as a control for the amount of protein loaded, were applied in different membranes (Millipore). Thereafter the secondary antibody peroxidase-conjugated (1:5000) was added. Films were developed and the intensity of the immunoreactive labeling was analyzed (Image analysis system, Image J, NIH Bethesda, USA). The values for each sample are expressed as a percentage of optical density (o.d) obtained using α-tubulin.

Corticosterone and ACTH Radio immuno assay (RIA)

Trunk blood was collected individually in labeled potassium-EDTA coated tubes (1.6mg EDTA/ml blood, Sarstedt, Germany), kept on ice and centrifuged for 15 minutes at 3000rpm at 4°C. Plasma was transferred to clean tubes and stored frozen at -20°C until the determination of corticosterone or ACTH by the MP Biomedical RIA kits (ICN, Biomedicals Inc., CA).

Data analysis and statistics

Data is expressed as mean ± standard error of the mean (SEM). Physiological and neuroendocrine parameters were analyzed by multifactorial ANOVA using SPSS software (version 7.5). Expression of molecular markers and behavioral measurements were analyzed by two way ANOVA followed by Bonferroni-adjusted post-hoc test using GraphPad Software (version 4). Differences were considered statistically significant at $p<0.05$.

Behavior of separate groups was tested in open field, elevated plus maze, forced swim and Morris water maze task (see supplementary data). For each behavioral task different groups of control and diabetic animals were used.

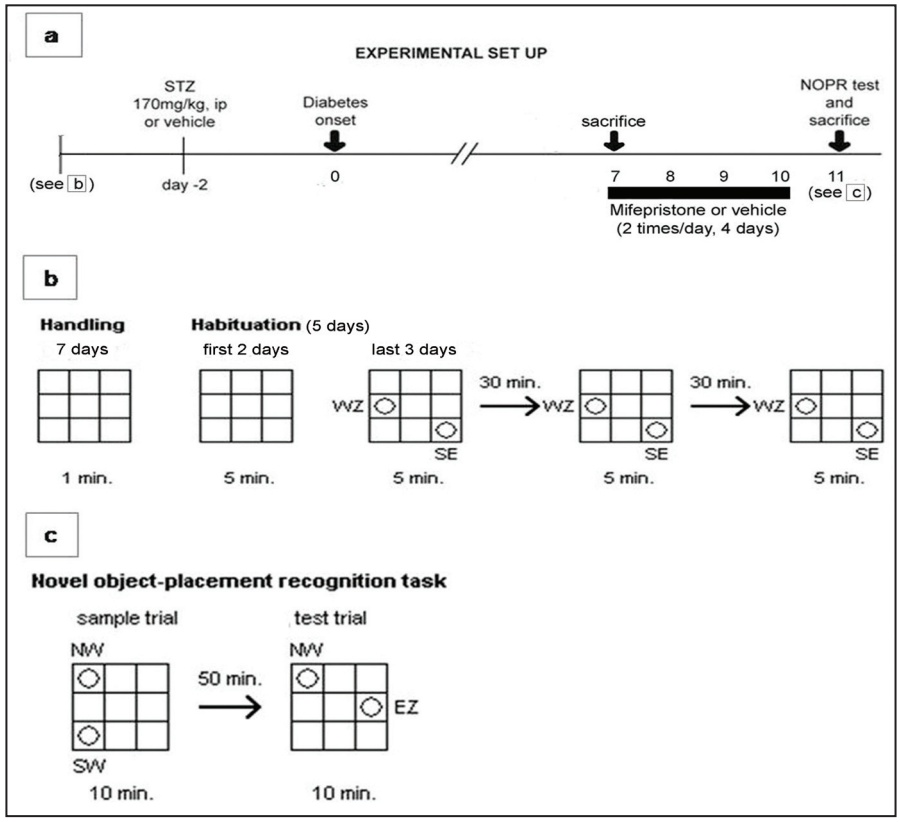

Figure 1*. Schematic representation of the methodology used.* a) *Experimental design.* b) *Handling and habituation for the NOPR task before STZ or vehicle injections.* c) *NOPR test trials at 11 days of diabetes.*

Results

Physiological and neuroendocrine parameters

Diabetic mice showed the characteristic clinical features of the disease after 6 days of diabetes. Multifactorial ANOVA revealed significant main effects of group (diabetic vs control: $F(8,30)$ 61.176 $p < 0.001$) and treatment with mifepristone ($F(8,30)$ 18.485 $p<0.001$ and a group x treatment interaction ($F(3,30)$ 5.191; detailed measurements and significances are given in Table 1). As we observed before (Revsin, *et al* 2008), diabetic mice showed hyperglycemia (>11mM), increased absolute adrenal weight and adrenal/body weight ratio, and decreased body weight, absolute thymus weight and thymus/body weight ratio. Furthermore, basal plasma corticosterone (CORT) levels were significantly increased, while ACTH concentration was significantly decreased compared to control mice. Untreated control and diabetic mice of 6 days of diabetes showed the same effects as the control and diabetic vehicle-treated groups, respectively (data not shown).

Mifepristone treatment increased adrenal weight, adrenal/BW ratio and CORT levels in control as well as in diabetic mice. In addition, this mifepristone-treated diabetic group showed increased ACTH and gain in BW, but thymus weight and thymus/BW ratio remained as low as in diabetic vehicle-treated mice (Table 1).

Table 1. *Effects of diabetes and mifepristone treatment on glycemia, body, adrenal and thymus weight, and plasma corticosterone and ACTH levels.*

	vehicle		mifepristone			
	control	diabetic	control	diabetic	Group	Group x treatment
Glycemia (mM)	6.8±0.23	27.8±1.3*	6.5±0.4	23.7±3.6$^{\$}$	138.1	ns
δ BW	3.1±0.3	-3.1±0.5	2.3±0.7	-0.01±0.5$^{\$/\#}$	51.4	11.7
Adrenal weight (mg)	1.5±0.1	2.6±0.1*	2.1±0.2*	3.1±0.2$^{\$}$	32.3	ns
Adrenal/BW ratio	57±2.5	134.7±6.5*	88.7±12.8*	135.6±15.2$^{\$}$	66	3.9
Thymus (mg)	22.9±0.7	7.7±0.7*	19.5±0.6	8.7±0.9$^{\$}$	322.2	12.6
Thymus/BW ratio	0.8±0.2	0.4±0.04**	0.8±0.04	0.4±0.04$^{\$}$	124.5	ns
CORT (ng/ml)	13.7±3.1	321.0±49.5*	297.0±79.3*	836.9±83.6$^{\$/\#}$	49.7	3.5
ACTH (pg/ml)	142.5±15	70.9±8.03*	133.0±22.5	148.8±17.4$^{\#}$	ns	6.9

$^{*}p<0.05$ vs control+vehicle, $^{\$}p<0.05$ vs control+mifepristone, $^{\#}p<0.05$ vs diabetic+vehicle. Values are expressed as mean ± SEM. δ BW: difference (in grams) in body weight at the time of vehicle or STZ injections and at sacrifice. Adrenal/body weight ratio is expressed as absolute weight x 1000 (gr)/ body weight (gr). CORT: corticosterone. F-values with df=1,37: between groups; df= 1 for all the group x treatment interactions measurements; only significant values are given; right column: significant group x treatment interactions. ns: non-significant

Mifepristone effects on the hippocampus

Astrogliosis

The number of astrocytes (GFAP+ cells, Figure 2A) in the *stratum radiatum* of the hippocampus was measured in six experimental groups: control and diabetic mice sacrificed 6 days after diabetes onset; control and diabetic mice treated with vehicle or mifepristone and sacrificed at day 11 of diabetes. Only at 11 days of diabetes was the number of astrocytes significantly increased (Figure 2). Treating diabetic mice with mifepristone for 4 days prevented the increase in the number of GFAP+ cells, which remained comparable to non-diabetic controls. Mifepristone treatment of non-diabetic mice did not modify this parameter.

c-Jun protein expression

Cellular activation was studied by Western blot using a specific antibody against c-Jun (Figure 3). To normalize c-Jun protein expression between groups, the endogenous non-regulated α-tubulin was measured. The optical density (O.D.) of c-Jun/α-tubulin ratio is shown in Figure 3A. The quantitative analysis reveals that hippocampal cellular activation takes place at 11, but not at 6 days after diabetes onset. Mifepristone treatment of non-diabetic mice did not alter c-Jun protein expression, but attenuated the c-Jun increase in diabetic mice. Comparable amounts of c-Jun protein expression were found between non-diabetic controls and mifepristone-treated diabetic mice.

*Cell proliferatio*n

Proliferative cells in the *dentate gyrus* (DG) were detected by the endogenous proliferation marker Ki-67. The Ki-67 antigen is a protein complex present only in the G_1, S, G_2 and M, but not the G_0 phase of the cell cycle (Gerdes, *et al* 1984; Endl and Gerdes, 2000). Clusters of Ki-67-positive cells were shown to be similar to BrdU after short survival time and were found almost exclusively in the subgranular zone (SGZ) (Kee, *et al* 2002; Oomen, *et al* 2007). Moreover, Ki-67 is a well-accepted proliferation marker (Gerdes, *et al* 1991; Heine, *et al* 2004; Krugers, *et al* 2007; Veenema, *et al* 2007).

After six days of diabetes, a significantly decreased cell proliferation in the SGZ of the DG was observed (diabetic day 6 vs control day 6, $p<0.05$, Figure 4). This reduction in the number of proliferative cells was maintained at 11 days of the disease: Ki-67 positive cell number of diabetic+vehicle group is significantly lower as compared to the control+vehicle ($p<0.001$). After mifepristone administration the number of Ki-67 positive cells is significantly increased in diabetic mice (diabetic+mifepristone vs diabetic+vehicle, $p<0.05$). Mifepristone treatment of control mice did not modify the Ki-67 positive cell number in comparison to the control vehicle-treated group.

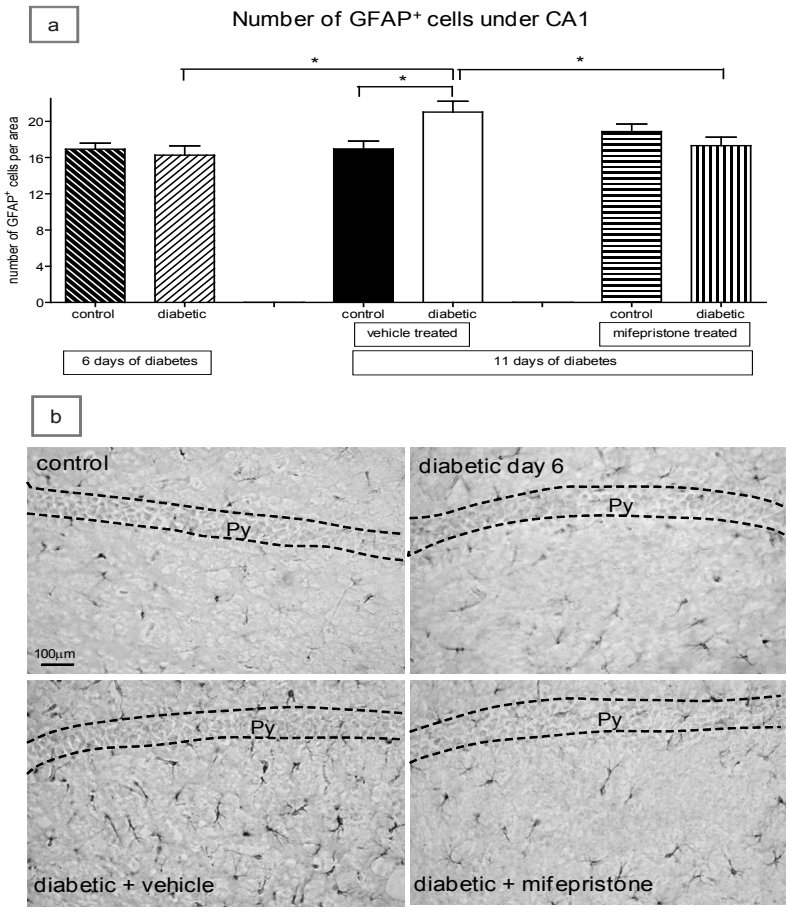

Figure 2. *Immunocytochemistry for GFAP.* a) *Quantification of GFAP+ cells number in the stratum radiatum of the hippocampus. Values expressed mean ± SEM, n=6-8, *p<0.05.* b) *Microphotographs of the different experimental groups. Py= pyramidal cells of the CA1 area. Control microphotograph correspond to a control + vehicle mouse.*

Diabetes effect on cognition

Only the more subtle hippocampal-dependent task, the novel object-placement recognition (NOPR), revealed significant impairments in cognitive performance of diabetic mice at 11 days of the disease. In the sample trial, the percentage of time that both objects are explored is similar in both groups (control: 50.51±5.34, diabetic: 57.84±3.84, expressed as percentage of preference for the object that will be re-located in the test trial, n=7-8). In contrast to mice of the control group, diabetic mice had no preference for the re-located object in the test trial (control: 61.37±5.54, diabetic: 44.41±4.98 %, of time exploring the re-located object, $p<0.05$).

Mifepristone effects on cognition. The NOPR task.

Locomotor activity

Locomotor activity expressed as distance moved, duration of the movement and mean velocity of the movement is presented in table 2. During the sample trial of the NOPR task, both vehicle- and mifepristone-treated diabetic mice walked slower and shorter distances, with shorter duration of movement when compared to control groups. Fifty minutes later, during the test trial, the diabetic vehicle-treated group was still less active. However, diabetic mifepristone-treated mice significantly increased their locomotor activity. Locomotor activity of control groups remained comparable in sample and test trials.

The latency to first exploration of the objects and to the zones, as well as the percentage of time in each zone did not differ between groups (data not shown). The number of mice at the beginning of the behavioral experiment was 12 per group. However, some diabetic and control+mifepristone mice died before the end of the experimental procedure. Moreover, two mice were discarded for the behavioral analysis from the diabetic+mifepristone group due to their inability to walk as a consequence of the metabolic deficits originated by the disease. The number of mice used was: control+vehicle n=12, diabetic+vehicle n= 10, control+mifepristone n= 8, diabetic+mifepristone n=6.

Spatial memory: exploration of the re-located object

During the sample trial, all groups spent a comparable percentage of time exploring both objects, expressed as percentage of preference for the object that will be re-located in the test trial (control + vehicle = 42.67±2.07, diabetic + vehicle = 49.47±5.17, control

***Table 2**. Effects of diabetes and mifepristone treatment on locomotor activity*

	vehicle		mifepristone	
Sample trial	control	diabetic	control	diabetic
Distance moved (m)	47.0 ± 2.5	22.0 ± 2.5*	49.5 ± 2	25.5 ± 4.7$^{\$}$
Mean velocity (cm/s)	8.0 ± 0.4	3.7 ± 0.4*	8.5 ± 0.4	4.3 ± 0.8$^{\$}$
Duration movement (s)	506 ± 9	335 ± 27*	533 ± 6	356 ± 35$^{\$}$
Test Trial				
Distance moved (m)	44.2 ± 2.5	22.0 ± 2.5*	46.3 ± 1.8	30.4 ± 2.4$^{\#}$
Mean velocity (cm/s)	7.4 ± 0.4	5.6 ± 0.5*	7.8 ± 0.3	6.7 ± 0.4
Duration movement (s)	490 ± 12	415 ± 17*	516 ± 10	474 ± 14$^{\#}$

**p<0.05 vs control+vehicle, $^{\$}$p<0.05 vs control+mifepristone, $^{\#}$p<0.05 vs diabetic+vehicle. Values are expressed as mean ± SEM. cm=centimeters, s= seconds.*

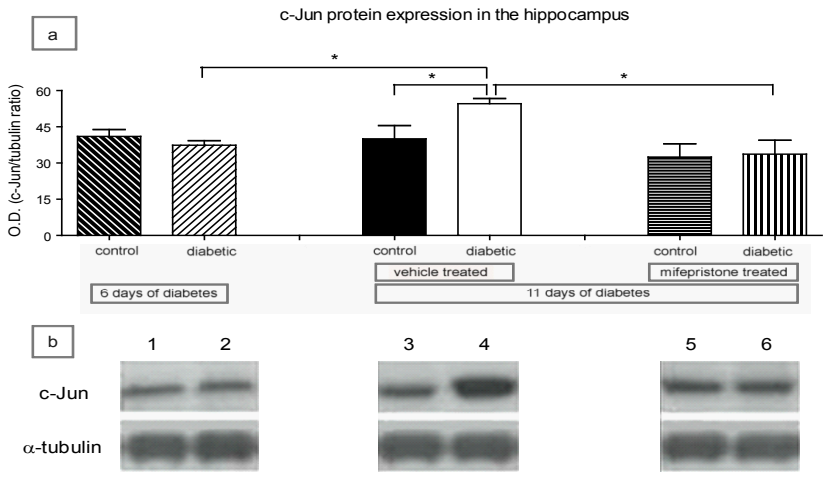

Figure 3. *Western blot for c-Jun.* a) *Quantification of c-Jun/α-tubulin optical density (O.D.) from hippocampal homogenates. Values expressed mean ± SEM, n=6, *p<0.05.* b) *Autoradiograph for c-Jun and α-tubulin. 1: control day 6; 2: diabetic day 6; 3: control+vehicle; 4: diabetic+vehicle; 5: control+mifepristone; 6: diabetic+mifepristone.*

+ mifepristone = 44.59±4.32, diabetic + mifepristone = 51.03±2.41). Figure 6 shows the percentage of preference for the object placed in the new location (i.e., the re-located object) during the test trial. As previously reported (Dere, *et al* 2005) the control vehicle-treated group preferred to explore the re-located object. The same effect was found in mifepristone-treated control mice. Conversely, diabetic mice explored both objects equally (50% of the exploration time for each object). The preference for the re-located object is restored when diabetic mice are treated with mifepristone. In addition, the difference in preference in this group is higher compared to its respective control ($p<0.05$, Bonferroni-adjusted post hoc test).

Discussion

Our results show that: (i) hippocampal integrity is threatened in STZ-treated diabetic mice as revealed by astrogliosis, increased cellular activation and decreased neuronal proliferation at 11 days of disease progression; (ii) diabetic mice show spatial memory deficits as is indicated by the impaired performance in a specific hippocampal-dependent task (the NOPR task); and (iii) 4-days of treatment with the GR antagonist mifepristone in the early phase of diabetes prevents the change in markers for hippocampal integrity and improves cognitive performance in the face of corticosterone hypersecretion

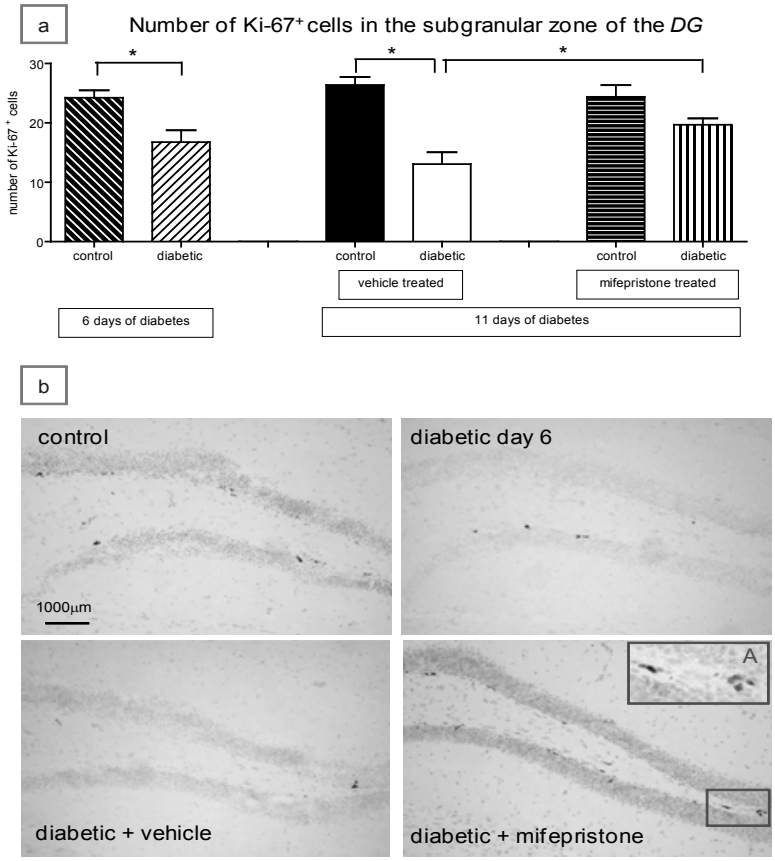

Figure 4. *Cell proliferation in the subgranular zone (SGZ) of the dentate gyrus. a) Quantitative analysis of the immunocytochemistry for Ki-67 in the SGZ. Values expressed mean ± SEM, n=6-8, *p<0.05. b) Microphotographs of the different experimental groups. Insert A: 4 times magnification of Ki-67+ cells. Control microphptograph correspond to a control + vehicle mouse.*

Mifepristone effects on hippocampal alterations of STZ-diabetic mice

Prevention of astrogliosis

Increased GFAP immunoreactivity representing astrogliosis in the hippocampus was previously reported at day 28 of STZ-diabetes in mice (Saravia, *et al* 2002). We found similar results already in 11-day diabetic mice, while GFAP immunoreactivity was not affected at 6 days of diabetes. Treatment with the GR antagonist mifepristone from day 6 to 10, prevented the GFAP increase at day 11, indicating that blockade of excess glucocortcoid activation of the GR can prevent hippocampal astrogliosis.

Reactive, GFAP-expressing astrocytes provide neuroprotection during metabolic

insults, stress or injury to nearby neurons by secreting growth factors, substrate-bound neurite promoting factors, and the removal of neurotoxins and excess glutamate (Kiessling, *et al* 1986; Liedtke, *et al* 1996; Lambert, *et al* 2000). Astrocytes may also protect neurons by increasing glucose uptake, metabolism and transport (Magistretti and Pellerin, 1999; Vesce, *et al* 1999), in addition to being necessary for the preservation of myelin and normal white matter architecture (Louw, *et al* 1998). Moreover, the close connection of astrocytes to the blood–brain barrier makes them early sensors of variations of glucose homeostasis, which can be communicated to neurons (Magistretti and Pellerin, 1999). The astrogliosis therefore can be considered to be a neuroprotective response to brain damage inflicted by excessive GR activation, which is prevented by the GR antagonist.

Prevention of cellular activation

We previously reported that T1D also resulted in cellular activation, as shown by an increase of c-jun- and c- fos-positive cells in neuronal populations of the CA1, CA3 and DG hippocampal areas (Revsin, el al 2005), and this is further supported by the current findings. While some authors imply a functional role of early gene products in neuronal cell death (Eilers, *et al* 2001; Barone, *et al* 2008), others suggested that they might rather contribute to cellular repair and/or regenerating processes (Herzog and Morgan, 1996; Herdegen, *et al* 1997; Waetzig, *et al* 2006). Since c-Jun was increased at day 11 but not at day 6 of STZ-diabetes, we conclude that either corticosterone caused the hippocampal cellular activation or that increased c-Jun-positive cells are a response to an emerging corticosterone-induced neuronal derangement in the hippocampus of diabetic animals, which is prevented by the GR antagonist.

Restoration of cell proliferation

Previously, we demonstrated a strong reduction in cell proliferation rate in STZ-diabetic mice (Saravia, *et al* 2004). The results shown in the present study confirm these previous observations and demonstrate that significant suppression of cell proliferation already occurs at early stages of diabetes, at day 6 and day 11, suggesting that newborn cells are particularly vulnerable to the detrimental effects of glucocorticoid excess. These data are in support of Stranahan *et al* (2008) showing that adrenalectomy and low-dose corticosterone replacement prevent the decreased proliferation and survival of newborn DG neurons in long term STZ-induced diabetes.

The data also show that GR blockade in diabetic mice restores the number of Ki-67 positive cells in the DG towards control levels. Therefore, the suppression of proliferation in diabetes is mediated directly and/or indirectly by glucocorticoids via the GR. In other studies neurogenesis was suppressed in high stress and corticosterone environments (Wong and Herbert, 2005; Mayer *et al*, 2006; Oomen *et al*, 2007), but not the Ki-67 marker observed in this study and by Stranahan *et al* (2008). Therefore, the proliferative response marker might be sensitive to a high corticosterone environment

during hyperglycemia and this effect is reversible by blockade of the GR. Nevertheless, the action of glucocorticoids on proliferation is likely to be indirect since newly formed cells in the DG have been found not to express corticosteroid receptors (MR and GR) (Cameron, *et al* 1993, Garcia, *et al* 2004, Wong and Herbert, 2005). Therefore, mature receptor-expressing dentate granule cells or glia surrounding the progenitors might determine proliferation and survival of these cells (Song, *et al* 2002; Hastings and Gould, 2003; Seki, 2003). However, a study from Garcia *et al* in 2004 showed that a small proportion of newly formed cells (15%) does in fact express GR 24 hrs after division.

In summary, the molecular changes described above indicate that mifepristone treatment interferes with the disruption of hippocampal integrity at early stages of STZ-diabetes. Whether mifepristone's prevention and/or reversion of the disturbances caused by diabetes is directly on hippocampal astrocytes, neurons and proliferative cells, or by changing its surroundings (i.e. their inputs) requires further investigation. It is worth noting that 4 days mifepristone treatment can affect not only the functioning of these cells (present study, Karst and Joëls, 2007; Wu, *et al* 2007; Mayer, *et al* 2006 and Wong and Herbert, 2005) but also the innervating projections from other brain regions (Witter, 2007). Moreover, in the present study, we reported that reversion of hippocampal alterations takes place in the face of hypercosticism. Recently, Oomen *et al* (2007) and Mayer *et al* (2006) reported similar beneficial effects of mifepristone in rats subjected to chronic unpredictable stressors and to high doses of exogenous corticosterone, respectively.

Cognitive performance

The previously observed HPA axis dysregulation in the type 1 diabetic mice (Revsin, *et al* 2008) suggested that hypercorticism and excessive GR activation might impair cognitive performance. Therefore, we assessed whether the learning and memory capabilities of STZ-induced diabetes mice were altered at different early states of the disease in a series of behavioral tasks (open field, forced swim test, Morris water maze and elevated plus maze). Surprisingly, diabetic and non-diabetic control mice performed equally well in these early stages of diabetes (Figures 1S, and 2S, 3S and Table 1S), while during a more prolonged state of diabetes behavioral deficits were established (Stranahan, *et al* 2008). It is possible that in our study these tasks are insensitive to the moderate hippocampal disturbances observed at early diabetes. For this reason, we decided to adopt another behavioral task for hippocampus-specific memory, the novel object-placement recognition (NOPR) task (Dere, *et al* 2005).

The NOPR task uses the spontaneous exploration of objects in novel locations (Li, *et al* 2004; Dere, *et al* 2005), and thus allows the study of mild hippocampal alterations. In accordance with the literature, non-diabetic control mice preferred exploring the object placed in the novel location. The diabetic mice, however, exhibit less exploration of the object in the novel location compared to controls (Figure 6). This reduced exploration

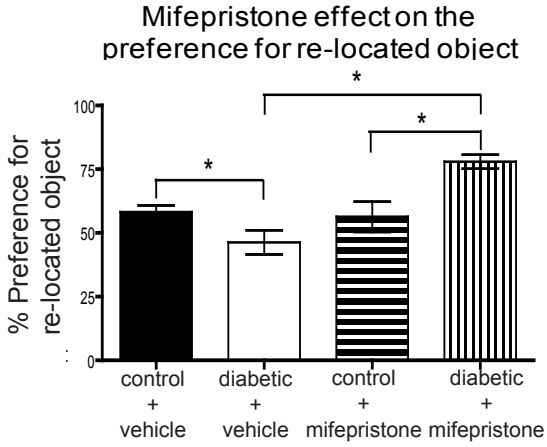

***Figure 5**. Mifepristone effect on the novel object-recognition task. Percentage of preference for the re-located object during the test trial is shown for control and 11-days diabetic mice vehicle- and mifepristone-treated. Values expressed mean ± SEM, *p<0.05 Bonferroni-adjusted post hoc test.*

displayed by the diabetic mouse indicates impaired spatial object-placement memory. It is note worthy that although diabetic mice showed a significantly decreased locomotor activity and exploration time of the objects than non-diabetic controls, the influence of these parameters on the preference for the objects was prevented by the method used to calculate the percentaje of preference for the re-located object.

Mifepristone restores spatial memory deficits of STZ-diabetic mice

Mifepristone treatment of diabetic mice restored the preference of these animals to explore an object in a novel location. This effect exerted by the GR antagonist occurred the day after termination of the treatment. In the studies of Stranahan *et al* (2008) the reinstatement in long term adrenalectomized T1D animals with exogenous corticosterone in physiological concentrations levels was a prerequisite to prevent deterioration of cognitive functions and impairment of LTP. The current study shows that similar results are achieved during the early stages of diabetes when the mild cognitive impairments are ameliorated by pharmacological blockade of the GR in an environment of extremely high circulating corticosterone. This observation raises a number of interesting issues:

First, the GR-mediated action of corticosterone on hippocampal function overrides the influence of insulin and glycemia (Stranahan, *et al* 2008). The current study also shows that chronic mifepristone causes extreme increases in corticosterone, and reverses some parameters (body weight, hippocampal function) but not others (thymus weight) (table 1; van Haarst, *et al* 1996). The treatment schedule was based on the efficacy of mifepristone in the treatment of psychosis (van der Lely, *et al* 1991) and psychotic depression (Flores, *et al* 2006), and previous animal studies using corticosterone injections and chronic stress (Mayer, *et al* 2006; Oomen, *et al* 2007). How precisely mifepristone exerts its lasting antagonistic central action through GR requires further investigation.

Second, the blockade with the antagonist becomes only effective when the GR's are fully occupied with high levels of corticosterone. At low levels of corticosterone GR is

hardly occupied, and GR blockade would be less effective (Ratka, *et al* 1989), and hence mifepristone is not effective in the non-diabetic controls.

Third, during GR blockade the MR is still freely accessible for corticosterone. In the NOPR test we specifically address MR-mediated functions, i.e. behavioral reactivity in the acquisition and retrieval of memory (Oitzl and de Kloet, 1992; Oitzl, *et al* 1994). Overexpression of MR could block some of the impairing behavioral effects mediated by GR (Ferguson and Sapolsky, 2007) and perhaps this explains why the GR antagonism in our diabetic mice not only rescued cognitive performance, but even enhanced it. GR antagonism also allows a more prominent function of the MR-mediated action in preserving integrity and stability of the hippocampus (Joëls, *et al* 2008). MR is neuroprotective (Lai, *et al* 2007), promotes neuronal survival and facilitates hippocampus function (Joëls, *et al* 2008). Hence this would predict that during GR blockade in the face of high circulating glucocorticoids the maintenance of hippocampal integrity is a necessary condition for improved performance in the NOPR task. Further experiments with administration of GR and MR antagonists are needed to test this possibility.

Fourth, understanding the mechanism would require the identification of the functional recovery of plasticity-related genes downregulated by the excess of glucocorticoids. Such genes are related to synaptic plasticity and glutamate transmission, alterations in dendrite and spine morphology, neurogenesis in the *dentate gyrus* and to the rapid functional responses underlying information processing in the NOPR test (Joëls, *et al* 2007; Morsink, *et al* 2007). Obviously, future research should be focused on the question how the molecular and cellular changes during diabetes and anti-glucocorticoid treatment are linked to behavior.

In conclusion, in the present study we have provided evidence that glucocorticoid excess and continuous activation of the GR compromise hippocampal integrity and function in T1D regardless of the hyperglycemic state. This deleterious effect of excess glucocorticoid can be abolished by a brief treatment with the GR antagonist mifepristone. The beneficial effect of mifepristone on molecular markers of hippocampal plasticity and spatial recognition is exerted in a high corticosterone environment and probably depends on blockade of excess GR activation, perhaps facilitated by more prominent MR-mediated actions preserving hippocampal integrity.

Supplementary materials and methods

Open Field

The squared open field (49.5 x 49.5 x 40 cm; gray polyvinylchloride) floor was virtually divided into nine quadrants of equal size resulting in 9 equal zones (see insert figure S1). Diffuse white light provided an illumination density of approximately 50 lux at the center of the open field. A radio was fixed in one corner of the room and provided background music. A cylinder (opaque gray 10 cm diameter; 20 cm high) was placed

in the center of the open field. The mouse was picked up at the base of the tail, placed into the cylinder. After 10 seconds, the cylinder was lifted and the mouse was allowed to explore the environment. The mouse was removed from the open field by a grid and returned to its home cage. After each trial, the apparatus was thoroughly cleaned with a 75% ethanol solution to remove or spread odor cues.

Forced swim test

One day prior to the forced swim test procedure, the locomotor activity of the mice was analyzed in order to discard locomotor differences during the swim test. At day 7 of diabetes, the mice were placed in a cylindrical container (16 cm diameter x 28 cm height) filled with 20.5 cm of warm tap water (25 ± 1 ºC) where it could not touch the containers floor. The duration a mouse spent climbing (strong mobility), swimming (mobility) and immobile in the cylindrical container for 6 minutes was recorded by a camera directly linked to the computer. After each session, the mice were placed under a red heating lamp for 10 minutes, thereafter replaced in their home cage, and the water was changed. In order to examine memory and memory capabilities, the day after the test was performed once more (re-test).

Morris water maze (MWM)

The pool, filled with warm tap water (26 ± 1 ºC), consisted of a 140 cm (diameter) x 40 cm (height) circular plastic tank with a removable circular Perspex platform 8.5 cm (diameter). The pool and platform were made white by using plastic tape. Non-toxic latex wall paint was added to the water to obscure the visual appearance of the platform. The water level was 1 cm above the platform for the non-visual trails. The pool was divided into four quadrants, and the light intensity of approximately 100 lux. Surrounding the pool a number of spatial cues were available like, plastic cubes and poster on the wall, a radio and the smell of other cages. After each trail the mice were placed under a red heating lamp for 5 minutes. After the last trail the mice were replaced in there home cage.
Training schedule:
- day 1 the mouse was placed in the MWM without a platform and had 2 minutes time to explore the maze (free swimming trial).
- days 2-4 the animals were placed, each day, once directly on the platform for 20 seconds.
- days 5-7 four consecutive trials, with 15 minutes interval between them, were conducted as follows: each mouse was placed in one of four start positions, which varies at each trial. A trial consisted in 1 minute to find the visual platform (1cm above the water). If it was not found it, the mouse was guided to the platform and let it sit on the platform for 10 seconds. Thereafter the mouse was placed under the heating lamp for 5 minutes, followed by the next trail.

- days 8-12 the mouse was placed in the MWM with a non-visual platform in a fixed position. The first three days there were 4 trials/day, the last two days there were 3 trails/ day.

After the training procedure, the mice were divided into 2 homogeneous groups based on the latency to find the platform and locomotor activity. 2 days after the last training day, seven mice were vehicle-injected (control group), and 8 mice were STZ-injected (diabetic group). At day 11 of diabetes, mice were test for memory and learning.
Test trials schedule (maximum of 1 minute each trial):
- day 1, trial 1, the non-visual platform was located at the same position as in training days 8-12. In the following 3 trials the platform position was changed.
- day 2 consisted in 3 trials where the platform location was the same as the day 1.

Supplementary results

Table 1S. *Effects of 11 days of diabetes on the elevated plus maze.*

Behavioral parameters	control	diabetic
% of duration in the open arm	24.5 ± 7.4	16.4 ± 6.0
% of duration in the close arm	75.5 ± 7.4	83.6 ± 6.0
Total number of times in open arm	4.5 ± 0.4	3.2 ± 0.9
Total number of times in close arm	5.5 ± 0.4	4.2 ± 0.9
% of duration walking	57.6 ± 8.0	48.8 ± 11.4
% of duration sitting	38.2 ± 8.7	48.5 ± 12.7
% of duration grooming	5.1 ± 1.3	5.0 ± 1.4
Total number of dipping	9.3 ±1.6	6.8 ± 2.3
Total number of stretch attempt	12.3 ± 1.7	5.7 ± 1.6*
Total number of rearing	12 ± 1.4	10 ± 2.3

**p<0.05. Values are expressed as mean ± SEM. Total duration of the task: 5 minutes*

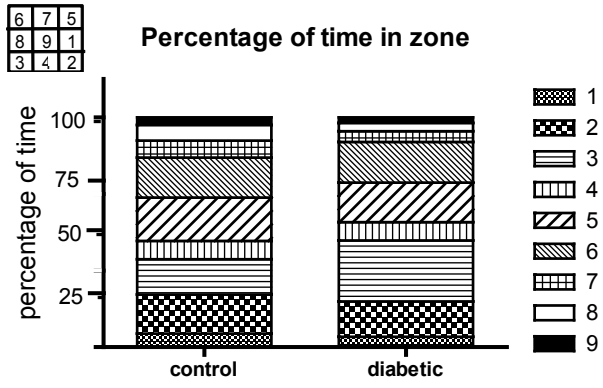

Figure 1S. *Diabetes effects on the open field task is represented as the percentage of time expending in each zone for control (n=12) and diabetic (n=10) mice. The latency for any other zone did not differ between groups (data not shown). Locomotor activity parameters are decreased in the diabetic group (data not shown) and are similar of those presented in table 2. Insert: schematic representation of the open field zones.*

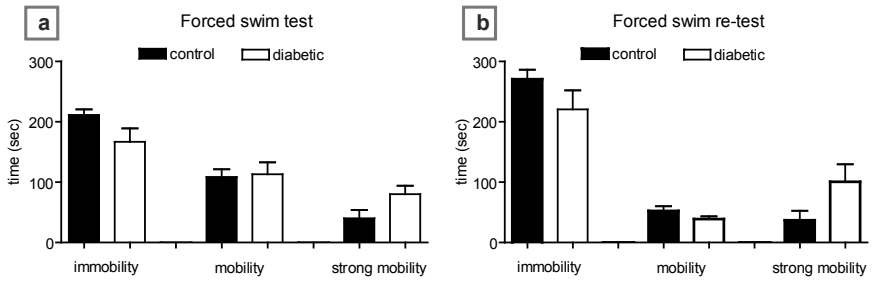

***Figure 2S**. Diabetes effects on the forced swim test. Three different behaviors were score as described before (Porsolt, et al 1977): immobility, mobility and strong mobility. Panel A shows the performance of control and diabetic animals the first day of the task (test). Panel B shows the performance 1 day after (re-test). Values are expresses as mean ± SEM., n= 6-7.*

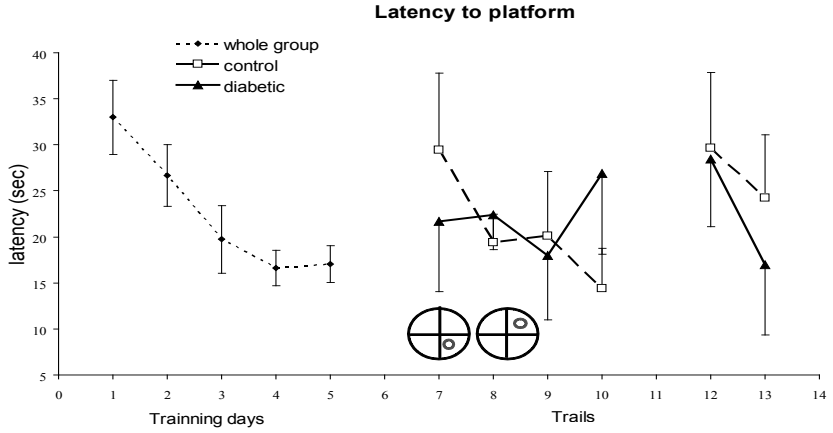

***Figure 3S.** Diabetes effects on the Morris water maze. Fifteen mice (whole group) were trained in the maze and subsequently divided into two homogeneous groups: control (vehicle-injected, n=7), and diabetic (STZ-injected, n=8). At day 11 of diabetes mice were test for memory assessment (trial 7, platform in the same location as in training), and for re-learning and memory (change of platform position, trial 8-13). Values are expresses as mean ± SEM. Inserts: schematic representation of the platforms location in trail 7 and 8-13.*

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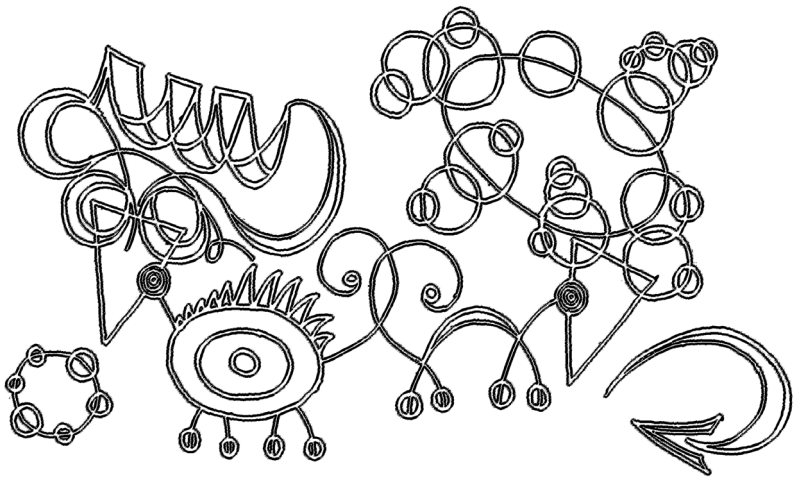
Chapter 6

GENERAL DISCUSSION



Outline

THE OBJECTIVE of the studies described in this thesis was to elucidate if glucocorticoids circulating in excess in mice suffering from type 1 diabetes enhance vulnerability to cerebral damage and cognitive impairment. For this purpose we have used two animal models, i.e. a pharmacological model, the streptozotocin (STZ)-treated mouse, and a genetic model the NOD mouse, which spontaneously develops type 1 diabetes. These models were used to test the hypothesis that the onset of diabetes induces first dysregulation of the HPA axis and subsequently hypersecretion of glucocorticoids which then renders the brain more vulnerable to metabolic insults causing damage and concomitant cognitive disturbances. Alternatively it also is possible that hyperglycemia and aberrant insulin levels cause deterioration of brain structure and function.

Main findings

The NOD model revealed a surge in ACTH release which likely preceded the onset and progression of diabetes marked by adrenal hyperreponsiveness and hypersecretion of corticosterone. To our surprise we found in the STZ model that not the initial ACTH surge was the most proximal cause of hypercorticism in diabetes, but rather the induction of adrenocortical ACTH receptors *per se*. At no time point after STZ administration ACTH levels did rise reinforcing the notion that hyperresponsiveness of the adrenals to ACTH may occur independent of the mitogenic activity of the peptide. In the same model, excess glucocorticoids rather than glycemia and insulin appeared causal to cerebral damage and mild cognitive impairment. These deficits in hippocampal function induced by high glucocorticoid concentrations were readily ameliorated by a brief treatment with the glucocorticoid receptor antagonist mifepristone.

HPA axis in a spontaneous T1D model, the NOD mouse

In the nonobese diabetic (NOD) mouse, a model of autoimmune type 1 diabetes (T1D), central nervous system alterations involve: (i) astrogliosis in the hippocampus during pre- and full-blown diabetes; (ii) decreased cell proliferation after diabetes onset, (iii) increased expression of hypothalamic arginine-vasopressin and oxytocin mRNAs and peptides in diabetes; and (iv) increased levels of circulating glucocorticoids. These results indicate hippocampal dysfunction and activation of the hypothalamus-pituitary-adrenal (HPA) axis in diabetes. Therefore, Chapter 2 tested the hypothesis that an altered HPA axis regulation in NOD mice may signal the onset and progression of the disease. Hence, we examined molecular markers of the hippocampal-HPA axis in diabetic and non-diabetic littermates. The results demonstrate that while the pre-diabetic phenotype seems to be characterized by adrenal hyporesponsiveness in view of high ACTH vs. low glucocorticoids levels, the opposite is observed in long-term diabetes. During full-blown T1D glucocorticoids are elevated and ACTH is significantly lower as compared

to non-diabetic littermates, a condition suggesting hyperresponsiveness of the adrenals to ACTH. Moreover, downregulation of the glucocorticoid receptor in the hippocampus and hypothalamus suggests that the capacity to suppress the HPA axis is disrupted. Such an impaired negative feedback would further promote hypercorticism.

The activation of ACTH release found in a subgroup of non-diabetic mice rises the question whether it precedes full-blown diabetes. Therefore, non-fasting C-peptide levels were determined, a measurement of residual beta-cell functionactivity, which is secreted in equimolar concentrations with insulin and is an appropriate outcome measure for T1D. We observed that the subgroup of non-diabetic mice exhibiting high ACTH levels also showed higher concentration of C-peptide as compared to the non-diabetic littermates with normal ACTH levels. In view of these results, it seems likely that the group of mice with elevated C-peptide might develop T1D. This indicates that ACTH release may precede the cascade of endocrine events triggered by the destruction of insulin-producing cells.

As mentioned above, ACTH hypersecretion occurs in a face of unaffected corticosterone levels, suggesting adrenal hyporresponsiveness and/or impaired function. This could facilitate the progression of autoimmunity. Cytokine levels, as an index of autoimmunity, can affect the HPA axis elevating CRH levels and destroying the adrenal glands. Although lymphocyte penetration of adrenals was established in NOD mice, no signs of immune destruction nor changes in corticosterone levels were found (Beales *et al*, 2002). Therefore, the idea that the lack of adrenal responsiveness is due to immune destruction of the adrenals in the subgroup of pre-diabetic NOD mice with high ACTH concentration can be discarded although a shift in the local cytokine balance cannot be excluded. However, our results from plasma cytokine concentrations (IL-1, IL-6 and TNFα) did not reveal an immune response neither at the time of the pre-diabetic ACTH surge nor during full-blown diabetes.

HPA axis alterations in type 1 diabetic mice

Type 1 diabetes is characterized by hypercorticism and lack of periodicity in adrenal hormone secretion. Our NOD mice data resemble the reported effects of food deprivation observed in young male mature rodents (Dallman *et al*, 1999). After food deprivation there is an immediate burst in ACTH possibly to sensitize the adrenals, subsequently hypercorticism is maintained in the face of very low circulating levels of insulin and ACTH suggesting a significantly enhanced adrenal responsiveness under these conditions. The hypercorticism reinforces catabolic activity and enhances the expression of peptides such neuropeptide Y which reinforce the drive for food seeking behaviour (Dallman *et al*, 1999). Likewise pups deprived from maternal care and feeding show a rapidly enhanced adrenal hyperreponsiveness which can be readily ameliorated when feeding is reinstated (Van Oers *et al*, 1998).

In Chapter 3 the data show that the expected rise in blood glucose levels induced by

STZ treatment preceded the surge in corticosterone secretion, which took place one day after diabetes onset. Surprisingly, there was no initial ACTH after STZ treatment. ACTH levels were below control levels during the first day after diabetes onset and remained low until day 11 during hypercorticism. These results suggest that in the STZ model sensitization of the adrenal glands to ACTH rather than the increase in circulating ACTH level characteristic for NOD mice is the primary event leading to hypercorticism. In support of this assumption, adrenal cell cultures of diabetic mice secrete higher amounts of corticosterone compared to controls, in response to ACTH (but not to vasopressin) and the adrenal glands increased expression of ACTH receptors (MC2 and MC5) in the adrenal glands.

To understand the regulation of the hypothalamic-pituitary-adrenal (HPA) axis activity that might lead to the adrenal hypersensitivity in diabetes, molecular markers were analyzed at different time points. We found that AVP mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus was increased from the day of diabetes onset. Hippocampal MR mRNA was initially up-regulated at the day of diabetes onset, but downregulated at day 11 of the disease. The decreased MR expression suggests that as a consequence the disrupted inhibitory regulation of the HPA axis contributes to the observed chronic hypercorticism. This result implies that a time-dependent adaptation to the new metabolic condition had occurred. Whether this adaptation in T1D leads to a more fragile state of the brain in which glucocorticoids excess may enhance the potential for damage and attenuate protective mechanisms, thus facilitating cognitive dysfunction and impairing the ability to respond to stress, is addressed in Chapters 4 and 5.

Discrepancies between the findings described above and published reports in relation to the HPA axis regulation have been found in different models of T1D. In a STZ-rat model Chan *et al* (2002) showed a profound activation of the HPA axis characterized by a marked increase in ACTH and corticosterone levels at 8 days after STZ injection. Moreover, AVP and CRH mRNAs expression in the hypothalamus and MR mRNA in the hippocampus were enhanced. Based on these results, the authors suggested that there is an increase in the central drive to the HPA axis that overrides the inhibitory influence of a negative feedback action by corticosterone. These authors and others (Scribner *et al* 1993) also showed that adrenal sensitivity is not increased in uncontrolled STZ-diabetic rats. No rise in corticosterone levels was found in diabetic animals after stimulation with low dose of ACTH.

Variability of the animals models used among studies could explain these disagreements: 1) STZ-rats and mice largely differ in survival (STZ-rats up to 8 months, STZ-mice few months); 2) severity of diabetes and routes of STZ administration and dosages (due to species variation in response to the drug). In particular in the studies from Chan *et al*, 10% sucrose in drinking water was given to the animals the first 24 hours following the STZ injection to prevent hypoglycemia, rendering moderately reduced fasting insulin levels. In our model, no sucrose was administered, which results in a condition characterized by hyperglycemia and low insulin levels at fasting and fed states.

In our mouse STZ model of T1D, the HPA axis readily reached a new setpoint characterized by high circulating corticosterone, low ACTH levels and enhanced adrenocortical sensitivity. The upregulation of ACTH receptors in the adrenal glands of STZ-induced diabetic mice might explain, at least in part, how hypercorticism is triggered and maintained. Moreover, the enhanced AVP mRNA in the PVN and decreased MR mRNA in the DG also may be considered manifestations of a profound disturbance in HPA axis regulation. A better understanding of these mechanisms may explain how diabetic pathophysiology causes adaptations in the CNS that may lead to an increased potential for damage and cognitive impairment

Hippocampal alterations in type 1 diabetic mice

Previous studies have demonstrated in models of type 1 diabetes (nonobese diabetic and streptozotocin (STZ)-treated mice), a marked astrogliosis and neurogenesis deficit in hippocampus and an increased expression of hypothalamic neuropeptides. In Chapter 4 the results are shown of a study designed to analyze the alterations of astroglia and neurons in the hippocampus of mice 1 month after STZ-induced diabetes. The STZ-diabetic mice presented: (a) increased number of astrocytes positive for apolipoprotein-E (Apo-E), a marker of ongoing neuronal dysfunction; (b) abnormal expression of early gene products associated with neuronal activation, including a high number of Jun positive neurons in CA1 and CA3 layers and *dentate gyrus*, and of Fos-expressing neurons in CA3 layer; (c) augmented activity of NADPH-diaphorase, linked to oxidative stress, in CA3 region. These data support the concept that uncontrolled diabetes leads to hippocampal pathology, which adjoin to changes in other brain structures such as hypothalamus and cerebral cortex.

Increased apolipoprotein-E astrocytic reactivity reflects an incipient damage as part of the rescue program to counteract neurodegeneration. Increased early gene expression proteins indicate emerging neuronal derangement in the hippocampus of diabetic animals. Elevated NADPH-diaphorase immunoreactivity indicates increased nitric oxide levels, which at high concentrations can cause neuronal death. These data indicate that hippocampal astrocytes and neurons are strongly activated one month after diabetes induction, exhibiting also higher oxidative stress. The results identify the hippocampus as a crucial brain structure sensitive to T1D disturbances. However, these findings also raise new questions: (i) are these changes indicators of damage caused by glucocorticoids, and (ii) do they lead to hippocampal dysfunction?

Glucocorticoids action on molecules and cognition in type 1 diabetes

A fundamental question in the central neuropathophysiology of T1D raised in Chapter 4 is whether glucocorticoid aggravate the morphological signs of hippocampal

neurodegeneration that herald the onset of cognitive impairment. Since hypercorticism *per se* can evoke a neurodegenerative cascade similar to the one observed in T1D, the role of glucocorticoid excess in STZ- diabetic mice in relation to morphological indices for neuronal viability and cognitive performance is described in Chapter 5. STZ-diabetic mice exhibit increased glucocorticoid secretion, hippocampal aberrations such as astrogliosis, increased c-Jun expression and decreased cell proliferation already 11 days after the onset of diabetes. At this time, retention and reversal learning in the water maze, retention in the forced swim task and emotional parameters in the elevated plus maze were not affected in diabetic mice. However, cognitive deficits became obvious in an exclusively hippocampal-dependent test, the novel object-placement recognition task. We showed that the continuous blockade of glucocorticoid action by treatment with the glucocorticoid receptor (GR) antagonist mifepristone (200 mg/kg p.o.) for 4 consecutive days (from day 7 to 10 of STZ-diabetes) prevented some of the hippocampal aberrations and reversed others. The decreased cell proliferation observed at day 6 was further decreased at day 11, but restored to control levels upon GR blockade. The prevention of hippocampal astrogliosis and increased neuronal activation showed that mifepristone treatment also can interfere directly with the progression of hippocampal alterations observed in diabetic mice. Moreover, when glucocorticoid action is inhibited by mifepristone administration, the cognitive deficits observed at day 11 were ameliorated. Surprisingly, the diabetic animal treated with mifepristone performed even better than the untreated controls.

The results presented in Chapter 5 provide evidence that glucocorticoid excess and the concomitant continuous activation of the GRs are responsible for molecular changes and hippocampal dysfunction at the behavioral levels at the early stages of diabetes. However, other factors altered in T1D might also be involved. Imbalance of glucose metabolism may be of importance in modulating the brain disturbances induced by diabetes. Some studies have described that hyper- and hypoglycemic episodes can cause acute cerebral dysfunction in diabetic animals (Biessels, *et al* 1994; Cryer, *et al* 1994). In the STZ-induced mouse model of T1D, GR antagonist normalized hippocampal functions regardless the hyperglycemic state, which is not altered in diabetic mice treated with mifepristone. Therefore, it is unlikely that hyperglycemia *per se* accounts for the hippocampal disturbances observed. These findings are in agreement with a recently published report (Stranahan *et al* 2008), in which corticosterone replacement of the adrenalectomized at physiological concentrations restored hippocampal dysfunctions, while glycemia remained elevated. However, the possibility of an effect of glucocorticoid excess on hippocampal glucose metabolism in T1D can not be discarded.

Furthermore, cerebral dysfunction in type 1 diabetes can also be as a result of insulin deficiency. Although published studies describe a central role of insulin in several animal models of diabetes (Sima and Li, 2005; Inouye, *et al* 2005; Chan, *et al* 2005; McNay, *et al* 2006), a recent report revealed that hippocampal impairments are not determined by changes in insulin production (Stranahan *et al*, 2008). The authors present evidence of normalization of hippocampal dysfunction with corticosterone replacement despite

profound differences in insulin levels (elevated insulin in a type 2 diabetes animal model, the db/db mice; and insulin deficiency in STZ-rats). Nevertheless, it is likely that the negative effect of diabetes on hippocampal plasticity may be attributable to an interaction between elevated glucocorticoids and insulin receptor signaling.

In Chapter 5 is described that astrogliosis, c-Jun expression and cell proliferation are normalized after GR blockade. Astrocytic functions suggest that astrogliosis emerges as a response to a brain damage and challenges to glucose homeostasis. Therefore, in a context where excess glucocorticoids have lost their function in restoring homeostasis and have become damaging to the brain, hippocampal astrogliosis can be viewed as an index for neuronal suffering. At the same time, increased Jun-positive cells indicate emerging neuronal derangement in the hippocampus of diabetic animals. These data together with the strong reduction in cell proliferation of diabetic animals indicate that disruption of hippocampal integrity may likely lead to the observed cognitive impairments. The normalization of these disturbances after a short treatment with the GR antagonist, demonstrates that excess glucocorticoid acting through GR causes the deficits in hippocampal function.

GR interact with MR in the hippocampus and both receptors mediate in a co-ordinate manner a differential and often antagonistic action of corticosteroids (Oitzl and de Kloet, 1992; Oitzl *et al*., 1995; Joëls, 1997; de Kloet *et al*., 1998). Therefore, MR and GR operate in balance in control of homeostasis and health. As a consequence of GR blockade the effect mediated by the higher affinity MR becomes more prominent. Hence, RU486 treatment blocks the 'damaging' excessive stimulation of GR in hippocampus, sparing protective MR-mediated actions underlying the enhanced cognitive performance (Oitzl *et al*, 1998).

Taken together, a new concept in diabetes has evolved from the data presented in this thesis and from a recent paper by Stranahan *et al* (2008): In this concept glucocorticoids play a causal role in diabetes neuropathology. Moreover, we revealed that the receptors for the glucocorticoids are crucial for the mechanism that underlies the disruption of hippocampal integrity and the impairment of cognitive performance. At the same time these receptors appear an excellent target for a therapy aimed to normalize the disturbed hippocampal functions characteristic for diabetes neuropathology.

Perspectives

Functional studies on human subjects have revealed that type 1 diabetic patients have a mild to moderate slowing of mental speed and diminished mental flexibility (Brands *et al*, 2005). From these studies, it has become evident that not all cognitive domains are equally affected. Diabetic humans show accelerated decline on tasks that require episodic memory and rapid information processing, whereas attention and language abilities are unaffected (Messier *et al*, 2005). Because episodic memory mainly requires temporal lobe structures, and language and attention primarily recruit other cortical and

prefrontal regions, these data suggest that the hippocampus is particularly vulnerable to the negative consequences of diabetes.

Some reports suggest that cognitive function in unstressed conditions is hardly affected by T1D (Jacobson *et al*, 2007). However, even mild cognitive defects can impact everyday activities in more demanding situations. In a pioneering study the role of cortisol in diabetes-induced cognitive deficits was investigated in humans (Sandeep *et al*2004). The authors showed an improved cognitive performance in diabetic humans after reduced bio-availability of excess cortisol through blockade of the enzyme 11β-Hydroxy-steroid-dehydrogenease type 1 which regenerates cortisol from its bio-inactive precursor. Sandeep *et al* concluded that hypercortisolism in diabetic patients may contribute to their hippocampal dysfunction.

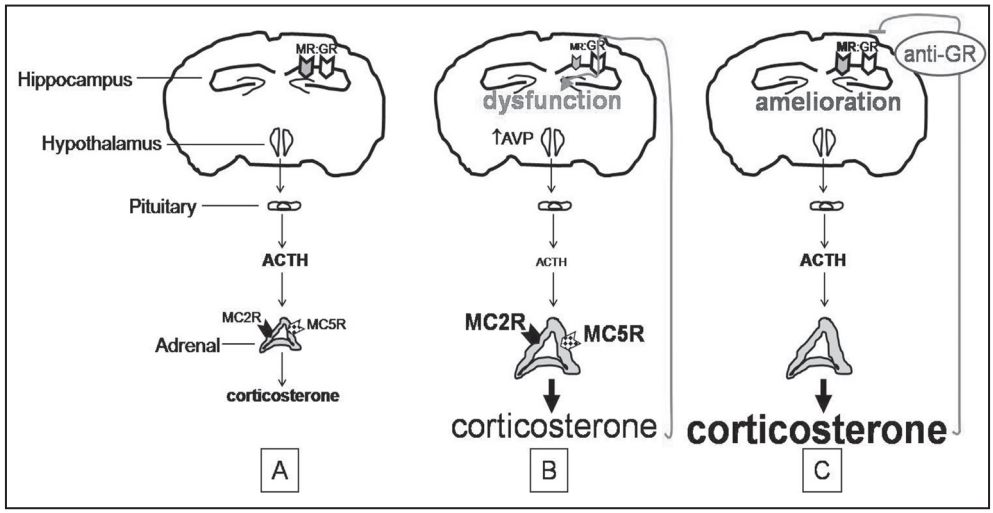


Figure 1. *Schematic representation of the main findings. Part A of the figure shows control conditions of the hippocampal-HPA axis. Part B summarizes the effects of STZ-induced diabetes: (i) hippocampal dysfunction characterized by alteration of molecular markers of hippocampal plasticity and cognitive impairments in a spatial memory task. (ii) Increased AVP mRNA expression. (iv) Significant decrease of ACTH levels. (v) Adrenal hypertrophy and increased ACTH receptors (MC2 and MC5R). (vi) Excessive corticosterone concentrations. These alterations indicate deteriorating hippocampal function leading to cognitive impairments, adrenal hypersensitivity to ACTH and HPA axis dysregulation. Part C illustrates the underlying mechanism of the hippocampal dysfunctions observed. Four days of anti-glucocorticods (mifepristone) treatment ameliorates hippocampal integrity and cognition. It is proposed that the mifepristone blockade of excessive hippocampal GR activation during diabetes restores the MR:GR balance underlying recovery from cognitive deficits. Anti-GR: anti-glucocorticods (mifepristone).*

In the current thesis two striking discoveries are described which both relate to glucocorticoids. Based on these findings we propose the following conceptual framework for the role of glucocorticoids in central pathology of type 1 diabetes (Figure 1).

First, while in NOD mice the expected pre-diabetic ACTH surge occurs, in the STZ model to our surprise the hypersecretion of glucocorticoids appears to be triggered by enhanced responsiveness of the adrenals to ACTH rather than that it depends on rising ACTH levels as primary event. In the STZ model he induction of ACTH receptors parallels the enhanced secretion of glucocortioids, while there is no rise in ACTH involved, which minimizes a role for the HPA axis as trigger. Second, we and others (Stranahan *et al*. 2008) have now clearly established that the onset and progression of cerebral damage and cognitive decline is due to the excess glucocorticoids circulating in diabetes. These signs of a damaged hippocampus function can be rapidly ameliorated with a brief anti-glucocorticoid treatment.

Since the intervention in corticosterone secretion and action now may become a treatment option it is essential to unravel the underlying mechanism of corticosterone action in the diabetic brain. One of the approaches is to study differentially regulated gene patterns in type 1 diabetes. The rationale would be that the action of circulating glucocorticoids in hippocampus is a key feature of stress as well as diabetes. However, there will be similarities and differences in genes turned on in either stress or diabetes. The differences may be related to reduced neuroprotection and increased damage-related gene expression, while in spite of the central role of glucocorticoids in diabetes also the action of insulin and glucose may be implicated; similarities will reflect overlap of structural remodeling of dendrites and suppression of neurogenesis that occur in both conditions and the fact that glucocorticoid actions are involved in both situations. To tip the protection/damage balance towards more severe impairment and degeneration in the hippocampus we will expose type 1 diabetic animals to daily stress. The outcome of these studies may lead to a conceptual framework explaining how type 1 diabetes may produce a more fragile state of the brain in which high levels of glucocorticoids enhance the potential for damage and attenuate a protective mechanism, which in concert would compromise adaptation and thus facilitate impairment of cognitive functions.

Another approach involves the evaluation of the hippocampal function in humans. It could be of interest to examine *post mortem* brains from male type 1 diabetes patients in comparison to aged match healthy individuals. Neurodegenerative markers similar to the ones used in rodents can be tested by immunocytochemistry and *in situ* hybridization. In addition, functional magnetic resonance imaging (fMRI) to measure the haemodynamic response related to neural activity in the brain, and behavioral tests to estimate cognitive performance in male patients and healthy subjects can be assessed. These results will make possible to understand better the impact of type 1 diabetes in the human brain and will allow us to proof the concept found from diabetic animals.

Conclusions

1) In a spontaneous model of type 1 diabetes, the NOD mouse, a surge in ACTH release may precede the onset of the disease. The subsequent HPA axis adaptations continue in overt diabetes with adrenal hypersensitivity leading to hypercorticism and poor shut-off of the stress response

2) In the STZ-induced diabetes mouse model hyperresponsiveness of the adrenal glands to ACTH, rather than an increase in circulating ACTH level, is the primary event leading to hypercorticism.

3) Type 1 diabetic mice show hippocampal pathology suggesting mild neurodegeneration and reactive nerve cell processes. Therefore, the hypercorticism observed in diabetic animals might enhance the vulnerability of brain areas with a high degree of plasticity such as the hippocampus.

4) Glucocorticoid excess is responsible for hippocampal disruption and cognitive impairment at the early stages of diabetes.

5) A brief treatment with GR-antagonist normalizes the markers for hippocampal dysfunction and hippocampus – related cognitive performance observed in T1D, indicating that the continuous GR activation is the likely mechanism by which glucocorticoids exert brain damage.

6) This action exerted by glucocorticoids and blocked by the GR antagonist involved blockade of GR activation, a process which makes the neuroprotective MR-mediated actions more prominent.

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SUMMARY-SAMENVATTING

Peripheral and autonomous neuropathies are well-known and devastating complications of type 1 diabetes (T1D). However, T1D can also impact the integrity of the central nervous system (CNS), and the reason why T1D affects CNS integrity remains to be elucidated. Studies on diabetic patients demonstrated mild to moderate slowing of mental speed and diminished mental flexibility (Brands *et al*, 2005). Although the alterations in cognitive functions under normal conditions are not severe, mild cognitive defects can influence everyday activities in more demanding situations. Indeed, in 2004 Sandeep *et al* reported that hypercortisolism in diabetic patients may contribute to their hippocampal dysfunction.

To investigate disease initiation, progression, and treatments without exposing humans to unnecessary and potentially unethical risks, animal models have been developed. The physiology of mice, rats, and other animals is remarkably conserved in comparison to the human condition, and over the last 40 years several animal models have become available. We have used two animal models, i.e. a pharmacological model, the streptozotocin (STZ) treated mouse, and a genetic model, the NOD mouse, which spontaneously develops type 1 diabetes. As type 1 diabetic patients, these animal models show high circulating glucocorticoid levels, increased sensitivity to stress, and morphological alteration in various brain areas (Fitzpatrick *et al*, 1993; Biessels *et al*, 1994; Saravia *et al*, 2001 and 2002; Revsin *et al*, 2005).

In the present study these models were used to test a general hypothesis that the onset of diabetes induces first dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis and subsequently hypersecretion of glucocorticoids which then renders the brain more vulnerable to metabolic insults causing damage and concomitant cognitive disturbances.

Chapter 2 tested the hypothesis that a defect in HPA axis regulation may signal the autoimmune response underlying diabetes onset. Hence, using the spontaneous model of T1D, the NOD mouse, we examined hippocampal-HPA axis markers. The findings revealed a surge in ACTH release without increased corticosterone in a sub-group of non-diabetic mice. Therefore, it is conceivable that this state of adrenal hyporesponsiveness facilitates autoimmunity to the beta-cells. Additionally, during full-blown diabetes ACTH is significantly lower in a face of corticosterone hypersecretion, suggesting a switch of hypo- to hyperresponsiveness of the adrenals to ACTH.

In Chapter 3 we investigated the underlying mechanism leading to hypercorticism in diabetes in the STZ-induced diabetes mouse model. The results showed that in this model hyperresponsiveness of the adrenal glands to ACTH, rather than an increase in circulating ACTH level as observed in the NOD mice, is the primary event leading to hypercorticism.

Based on the involvement of the hippocampus on the HPA axis regulation, Chapter 4 analized whether hippocampal dysfunction occurs in diabetes. We demonstrated that diabetic mice show hippocampal pathology suggesting mild neurodegeneration and reactive nerve cell processes.

In Chapter 5, we further elucidated whether the hypercorticism observed in diabetic animals might enhance the hippocampal dysfunction. Using a brief treatment with the glucocorticoid receptor (GR)-antagonist mifepristone we analyzed hippocampal markers and hippocampus–related cognitive performance in T1D. The data indicated that mifepristone administration normalizes hippocampal dysfunction and the impaired cognition of diabetic mice. This finding supports the mounting evidence that glucocorticoid excess is responsible for hippocampal disruption and cognitive impairment at the early stages of diabetes via continuous and excessive GR activation.

Taken together, a new concept in diabetes has evolved from the data presented in this thesis, in which glucocorticoids play a causal role in diabetes neuropathology. Moreover, we revealed that the receptors for the glucocorticoids are crucial for the mechanism that underlies the disruption of hippocampal integrity and the impairment of cognitive performance. At the same time these receptors appear an excellent target for a therapy aimed to normalize the disturbed hippocampal functions characteristic for diabetes neuropathology.

Cognitieve stoornissen zijn geassocieerd met diabetes en het gaat hierbij met name om stoornissen in de functie van de hippocampus, een hersengebied dat een prominente rol speelt in leer- en geheugenprocessen. Hoe deze cognitieve stoornissen tot stand komen bij een verstoorde glucosebalans is niet bekend. In het onderzoek beschreven in dit proefschrift is de hypothese getoetst dat glucocorticoïden afgegeven door de bijnier de diabetes neuropathologie veroorzaken. Deze hypothese is gebaseerd op de sterk verhoogde bloedspiegel van het glucocorticoïd corticosteron, dat vergelijkbare hersenschade en cognitieve stoornissen kan veroorzaken als waargenomen is bij diabetes.

Om dit doel te realiseren is een tweetal diermodellen gebruikt: een model voor de spontaan Type 1 diabetische muis, de z.g. non obese diabetes (NOD) muis, en de muis waarbij Type 1 diabetes is opgewekt middels toediening van streptozotocine (STZ), waarin de betacellen chemisch vernietigd worden. De volgende resultaten staan in het proefschrift en zijn reeds, of worden binnenkort, gepubliceerd in internationale tijdschriften.

In hoofdstuk 2 wordt onderzoek beschreven ontworpen om de hypothese te toetsen dat een defect in de de hypothalamus-hypofyse-bijnier (HHB) as de autoimmuniteit op gang brengt die ten grondslag ligt aan de afbraak van de beta cel, die dus uiteindelijke tot diabetes leidt. Dus, door gebruik te maken van de NOD muis, is inderzoek verricht naar de hippocampus-HHB interactie. Tot onze verrassing kon een subgroep van niet-diabetes dieren geïdentificeerd worden met een buitensporige spiegel aan ACTH zonder dat corticosterone niveaus verhoogd waren. Het is derhalve denkbaar dat deze toestand van verminderde bijnierschorsgevoeligheid voor ACTH de autoimmuniteit tegen de betacel bevordert. Voorts, vindt er tijdens ‘full-blown diabetes een omslag plaats naar een toestand gekenmerkt door lage ACTH gehaltes met een sterk verhoogd corticosteron, hetgeen een omslag van hypo- naar hyperresponsiviteit van de bijnier voor ACTH betekent.

In hoofdstuk 3 is het streptozotocine (STZ) model onderzocht. In de STZ muis blijkt juist verhoogde gevoeligheid van de bijnier voor ACTH het ontstaan van diabetes te markeren. Deze verhoogde gevoeligheid blijkt ook uit de toename van het aantal ACTH receptoren in de bijnierschors na toediening van STZ.

In hoofdstuk 4 wordt onderzocht of de hippocampus is aangetast. Inderdaad blijkt al na enige dagen dat markers voor celschade, en neuronale en gliacel-activatie in respons op de celschade zijn toegenomen.

In hoofdstuk 5 is onderzoek beschreven om vast te stellen of het hypercorticisme kenmerkend voor diabetes naast schade in de hippocampus ook de functie van de hippocampus beïnvloed heeft. Inderdaad zijn de ratten gestoord in de uitvoering van een ruimtelijke leertaak waarvoor een goed functionerende hippocampus nodig is. Vervolgens is nagegaan of met een korte behandeling met de glucocorticoïd antagonist mifepristone de beschadigingen en functieverlies omkeerbaar zijn. De gegevens tonen aan dat inderdaad hippocampusschade en cognitieve stoornis teniet gedaan kan worden met deze antagonist. Deze ontdekking geeft steun aan een groeiend aantal gegevens die er op

wijzen dat een overmaat aan glucocorticoïden een causale rol speelt in neuropathologie van de hippocampus via een continue en overmatige stimulatie van de glucocorticoïd-receptor (GR).

In conclusie, dit promotie onderzoek heeft geleid tot een nieuw concept van de neuropathologie van diabetes, waarin glucocorticoïden uit de bijnier een causale rol spelen. Bovendien, blijkt dat de receptoren voor glucocorticoïden van cruciale betekenis zijn voor het mechanisme dat ten grondslag ligt aan de verstoring van hippocampale integriteit en de cognitieve stoornissen. Tevens blijken deze receptoren een uitstekend aanknopingspunt op te leveren voor een therapie die er op gericht is verstoorde hippocampusfuncties te herstellen die kenmerkend zijn voor diabetes neuropathologie.

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Curriculm Vitae

Yanina Revsin was born on September 4th, 1975 in Buenos Aires, Argentina. She attended secondary school at the Instituto Libre de Segunda Enseñanza (ILSE) in Buenos Aires and she graduated in 1993. In 1994 she commenced the study of biology at Buenos Aires University (UBA). In 2000 she defended the master thesis "*Neurochemical Alterations in Experimental Diabetes Mellitus"* performed at the Laboratory of Neuroendocrine Biochemistry at the Institute of Biology and Experimental Medicine (IBYME-CONICET) under the supervision of Prof. Dr. Alejandro F. De Nicola and Dr. Flavia E. Saravia. Till 2003 she worked as research assistant in the same laboratory continuing the studies on the impact of type 1 diabetes on the central nervous system, and specializing in neuroendocrinology. In the same year she started the PhD studies described in this thesis at the Division of Medical Pharmacology, Leiden University. This work was supervised by Prof. Dr. E. Ronald de Kloet and Alejandro F. De Nicola and co-supervised by Prof. Dr. Melly S. Oitzl.

Yanina Revsin nació el 4 de Septiembre de 1975 en Buenos Aires, Argentina. Ella asistió al colegio secundario en el Instituto Libre de Segunda Enseñanza (ILSE) en Buenos Aires, graduándose en 1993. En 1994 ella comenzó el estudio de biología en la Universidad de Buenos Aires (UBA). En el año 2000 presentó su tesis de licenciatura titulada *"Alteraciones Neuroquímicas en Diabetes Mellitus Experimental"* desarrollada en el laboratorio de Bioquímica Neuroendocrina en el Instituto de Bilogía y Medicina Experimental (IBYME-CONICET) bajo la supervisión del Prof. Dr. Alejandro F. De Nicola y la Dr. Flavia E. Saravia. Hasta el año 2003 ella permaneció en el mismo laboratorio continuando con los estudios sobre el impacto de la diabetes tipo 1 en el sistema nervioso central, y especializándose en neuroendocrinología. En el mismo año comenzó sus estudios de Doctorado descriptos en ésta tesis en la División de Medicina Farmacológica de la Universidad de Leiden en Holanda. Este trabajo fue realizado bajo la supervisión del Prof. Dr. E. Ronald de Kloet y el Prof. Dr. Alejandro F. De Nicola, y co-supervisado por la Prof. Dr. Melly S. Oitzl.

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Yanina

“ What makes a scientist important is how well he or she has penetrated into the unknown.“

Claude Bernard